

# Hormone-Sensitive Lipase Purification from Adipose Tissue, Properties and Function

Gudrun Fredrikson



Lund 1984



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| <b>Abstract</b><br>Hormone-sensitive lipase catalyzes the rate-limiting step in the hydrolysis of the triacylglycerols stored in adipose tissue. The activity of the enzyme is regulated, through reversible phosphorylation, by e.g. noradrenaline, ACTH, glucagon and insulin. After removal of lipids from rat adipose tissue extracts by ultracentrifugation the enzyme was solubilized with non-ionic detergent and purified 2000-fold to approx. 50% protein purity by repeated gradient sievortptive chromatography on QAE-Sephadex, and affinity chromatography on immobilized triacylglycerol. The enzyme was concentrated and the detergent exchanged on hydroxylapatite. It had an $M_r=84000$ , a molar specific activity of 600 mol/s/mol against diacylglycerol and catalyzed the hydrolysis of tri-, di- and monoacylglycerol and cholesterol ester at the relative rates of 1:10:4:1.5, with a marked preference for the 1(3)-ester bonds of the acylglycerol substrates. The lipase was inhibited by micromolar diisopropyl fluorophosphate (indicating a serine residue in the active site) by NaF, $HgCl_2$ and detergent. It had amphiphilic properties but it is not known if the enzyme is in membranes in the intact fat cell. Hormone-sensitive lipase from swine adipose tissue was purified to 10% purity according to the same protocol. The swine enzyme had identical or very similar properties but a slightly more acidic pI. Complete hydrolysis of triacylglycerols is not achieved by hormone-sensitive lipase alone, but monoacylglycerol lipase is needed to hydrolyze the monoacylglycerols produced. The neutral cholesterol ester hydrolase of adrenal cortex was found to have several properties, including subunit size, specific activity, substrate specificity, inhibition characteristics and phosphorylation/activation, in common with hormone-sensitive lipase. It is likely that they are actually the same enzyme with separate functions in the respective tissue, depending on the substrates available. |                                                      |                              |
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HORMONE-SENSITIVE LIPASE  
PURIFICATION FROM ADIPOSE TISSUE, PROPERTIES AND FUNCTION

AKADEMISK AVHANDLING

som för avläggande av doktorsexamen vid medicinska fakulteten  
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Gudrun Fredrikson  
fil.kand., Sm

Lund 1984



From the Department of Physiological Chemistry,  
University of Lund, Sweden

# Hormone-Sensitive Lipase Purification from Adipose Tissue, Properties and Function

Gudrun Fredrikson

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Lund 1984

"....dansen går med små, småbitte skridt  
og vi ved at dansen ikke standser  
vi får nye sko når de gamle de er slidt"

De små skridts tunge dans  
Trille

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## LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their roman numerals.

- I. Synthesis of  $^{14}\text{C}$ -labelled alkyl polyoxyethylene detergents. (1982)  
G. Fredrikson, L. Krabisch and P. Belfrage  
J. Lipid Res. 23, 1246-1248
- II. Hormone-sensitive lipase of rat adipose tissue. Purification and some properties. (1981)  
G. Fredrikson, P. Strålfors, N.Ö. Nilsson and P. Belfrage.  
J. Biol. Chem. 256, 6311-6320
- III. Positional specificity of hormone-sensitive lipase of rat adipose tissue. (1983)  
G. Fredrikson and P. Belfrage  
J. Biol. Chem. 258, 14253-14256
- IV. Hormone-sensitive lipase from swine adipose tissue: identification and some properties.  
F.-T. Lee, S.J. Yeaman and G. Fredrikson, P. Strålfors and P. Belfrage.  
Comp. Biochem. Physiol. In press.
- V. Roles of hormone-sensitive lipase and monoacylglycerol lipase in adipose tissue lipolysis.  
G. Fredrikson, H. Tornqvist and P. Belfrage  
In manuscript.
- VI. Direct evidence that cholesterol ester hydrolase of adrenal cortex is the same enzyme as hormone-sensitive lipase from adipose tissue. (1982)  
K.G. Cook, S.J. Yeaman and P. Strålfors, G. Fredrikson, and P. Belfrage.  
Eur. J. Biochem. 125, 245-249

## ABBREVIATIONS

ACTH, adrenocorticotrophic hormone

DFP, diisopropyl fluorophosphate

SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis

cmc, critical micellar concentration

QAE-, diethyl-(2-hydroxypropyl) aminoethyl-

## INTRODUCTION

Triacylglycerols are stored in adipose tissue of mammals at times when there is an excess of energy intake. When the energy intake is not sufficient for the needs of the organism, the stored triacylglycerols are hydrolyzed, and fatty acids and glycerol released to be used as energy source by other tissues. The key enzyme in the control of lipolysis is hormone-sensitive lipase. The rate of lipolysis is controlled through hormonal and nervous mechanisms. Depending on the species, adrenaline, nor-adrenaline, adrenocorticotrophic hormone (ACTH) and glucagon increase the rate, whereas insulin causes a decrease.

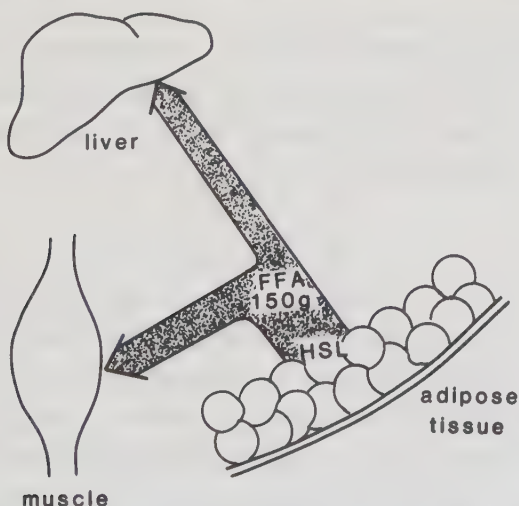


Fig. 1. The flow of fatty acids from adipose tissue, where hormone-sensitive lipase controls the rate of release, to the main sites of utilization, skeletal muscle and liver. FFA, free fatty acids; HSL, hormone-sensitive lipase.

While the uptake of fat from the intestine is approx. 100 g per 24 hours in man, the release from adipose tissue is approx. 150 g per 24 hours (Fig. 1), approaching half of the daily energy

conversion. This illustrates the central role of hormone-sensitive lipase in the organism, comparable to the role of glycogen phosphorylase in the glucose metabolism.

In adrenal cortex steroid hormones are synthesized. The cholesterol back-bone used in this process is derived from cholesterol/cholesterol esters in the plasma lipoproteins. Excess cholesterol is stored as cholesterol esters in the tissue. When cholesterol is needed for steroid hormone synthesis the stored esters are hydrolyzed by cholesterol ester hydrolase, one of the rate limiting steps in steroid hormone biosynthesis. The rate of steroid hormone synthesis is controlled by ACTH.

Hormone-sensitive lipase of adipose tissue and cholesterol ester hydrolase from adrenal cortex, and their regulation have recently been reviewed (Belfrage et al., 1984; Strålfors and Belfrage, 1984; Belfrage, 1984).

## BACKGROUND

Hormone-sensitive lipase as a term for the lipolytic activity of adipose tissue that responded to hormones was first used by Vaughan and coworkers (1964). The existence of such a hormone-stimulated lipolytic activity in rat adipose tissue extracts had previously been reported by others (Björntorp and Furman, 1962; Hollenberg et al., 1961; Rizack, 1961), but Vaughan et al. (1964) were the first to give a more detailed description. Hormone-sensitive lipase was described as a lipolytic activity which had a pH-optimum around 7, was inhibited by diisopropyl fluorophosphate (DFP), and its activity could be increased by prior incubation of the tissue with hormones, e.g. noradrenaline, ACTH, and glucagon (Vaughan et al., 1964). Hormone-sensitive lipase was distinguished from lipoprotein lipase by these criteria, and also from a distinct monoacylglycerol lipase, with a pH-optimum of 8, also

sensitive to DFP, but little, if at all, stimulated by hormones (Vaughan et al., 1964). The enzyme activity was further characterized (Gorin and Shafrir, 1964; Hollett, 1964; Rizack, 1964; Schwartz and Jungas, 1971) and several attempts were made to purify the enzyme (Strand et al., 1964; Tsai et al., 1970). A problem in the work with the lipase was its tendency to associate with tissue lipids. Attempts to delipidate the lipase with organic solvents were not successful, since the lipase was not stable to such treatment (Strand et al., 1964; Tsai et al., 1970). A major breakthrough was the partial purification of rat adipose tissue lipase by Huttunen et al. 1970 (Huttunen et al., 1970b). They described how most of the tissue triacylglycerols could be removed by spinning a tissue homogenate, in 0.25 M sucrose, at 100,000 x g. In 0.15 M KCl, which had been used previously, most of the lipase remained bound to the floating lipid layer, but in 0.25 M sucrose it was recovered in the supernatant (Huttunen et al., 1970b). The activity of the lipase in the supernatant was rapidly lost, but the addition of EDTA prevented this inactivation (Huttunen et al., 1970b). Precipitation of the enzyme at pH 5.2 resulted in a more concentrated preparation and this pH 5.2 precipitate fraction was stable for long periods of time (Huttunen et al., 1970b).

Most of the early studies on the properties of hormone-sensitive lipase have been performed on pH 5.2 precipitate fraction. Huttunen et al. (Huttunen et al., 1970b) also confirmed the previously reported inhibition of the lipase by NaF (Rizack, 1961). This inhibition has since then been used as one of the criteria to distinguish hormone-sensitive lipase from lipoprotein lipase (see e.g. Tornqvist et al., 1978b).

Huttunen and coworkers also reported a partial purification of hormone-sensitive lipase from rat adipose tissue (Huttunen et al., 1970a) using differential, preparative ultracentrifugation.





The enzyme was 100-fold purified to a final specific activity of 1  $\mu\text{mol}$  fatty acid produced per min and mg protein (Huttunen et al., 1970a). The enzyme preparation, however, contained phospholipids to about half its weight, and the lipase resided in large complexes with lipids and other proteins, having an average molecular weight of about  $7 \cdot 10^6$  (Huttunen et al., 1970a). The lipase was sometimes detected in smaller complexes (about  $0.5 \cdot 10^6$ , Pittman et al., 1972), for unknown reasons.

A major breakthrough in the work with isolated rat adipose tissue hormone-sensitive lipase was the partial purification and identification of the enzyme protein reported by Belfrage et al. (1977). The enzyme was identified as an  $M_r=86000$  polypeptide by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Belfrage et al., 1977). The lipase was solubilized from tissue lipids by non-ionic detergent and could then be partially purified by conventional protein purification procedures in the presence of the detergent. The enzyme activity was followed during the purification by its activity towards a diacylglycerol analogue substrate (Belfrage et al., 1977; Tornqvist et al., 1978a). The activity of the lipase towards this substrate is much higher than towards triacylglycerols (Belfrage et al. 1977). Activity towards triacylglycerols could hardly be detected after dilution during the chromatographies, especially since the enzyme activity was depressed in the presence of detergent (Belfrage et al., 1977).

Khoo and coworkers in 1974 started to use hen adipose tissue as a source of hormone-sensitive lipase (Khoo and Steinberg, 1974). The main reason for this was that the enzyme activity was increased 10-fold after incubation with cAMP, ATP and protein kinase (i.e. conditions under which the rat enzyme was activated only 2-fold). Hormone-sensitive lipase of hen adipose tissue was shown to be immunologically distinct from lipoprotein lipase

(Khoo et al., 1976). Partial purification of hormone-sensitive lipase from human adipose tissue has also been reported (Verine et al., 1976), although no substantial purification was obtained.

Besides hormone-sensitive lipase, adipose tissue contains several other lipolytic enzymes: lipoprotein lipase, lysosomal acid lipase and monoacylglycerol lipase. The role of lipoprotein lipase is to hydrolyze the triacylglycerols in the plasma lipoproteins. The fatty acids can then be taken up in the peripheral tissues and used as a source of energy or for storage (adipose tissue) (for recent reviews see e.g. Olivecrona and Bengtsson, 1984; Smith and Pownall, 1984). Lysosomal acid lipase has its function in the intracellular lipid metabolism (for review see Fowler and Brown, 1984), but it is not involved in the hormone-sensitive lipolysis of stored lipids (Belfrage et al., 1984). Monoacylglycerol lipase (Tornqvist and Belfrage, 1976a), which has a high activity towards monoacylglycerols but virtually none towards higher acylglycerols has been proposed to have a role in the degradation of monoacylglycerols resulting from both lipoprotein lipase and hormone-sensitive lipase action (Tornqvist et al., 1978b; recent review Belfrage et al., 1984). All these enzyme activities can be differentiated from each other on the basis of their different pH-optima, inhibition characteristics and substrate specificities.

Adipose tissue from rat contains only approx. 2  $\mu$ g hormone-sensitive lipase per g tissue (II). A preliminary aim of the present study was to develop methods for the purification and handling of the enzyme from this tissue source, at a preparative scale. A secondary aim was to use the purified enzyme to establish the identity of the enzyme protein and examine those enzymological and physicochemical properties which could be studied with the limited amounts of enzyme made available by this purification.

The purified enzyme has also been used in other studies (Nilsson, 1982; Strålfors, 1983) to examine the mechanism of regulation of the enzyme activity by reversible phosphorylation, both with the isolated enzyme and in intact adipocytes. These aspects will not be discussed in this thesis.

#### DETERGENT SOLUBILIZATION OF HORMONE-SENSITIVE LIPASE

Over the past decade detergents have found increasing use to solubilize intrinsic membrane proteins (Helenius and Simons, 1975; Tanford and Reynolds, 1976). When the concentration of detergent is sufficiently high, lipids and proteins are dissolved in detergent micelles with only one protein molecule per micelle (Helenius et al., 1979; Fig. 2). The membrane proteins can then be purified by conventional protein purification methods (Hjertén et al., 1981), in the presence of detergent.

There are several different kinds of detergents available (Helenius and Simons, 1975; Helenius et al., 1979). Of these, non-ionic detergents of the alkylpolyoxyethylene ether type are often used since they are mild and usually solubilize proteins without denaturation (Helenius and Simons, 1975; Helenius et al., 1979), and since they do not interfere with fractionations based on protein charge. Alkylpolyoxyethylene ether detergents have the general formula  $C_nH_{2n+1}(OCH_2CH_2)_xOH$ , abbreviated  $C_nE_x$ . In commercially available detergents the polyoxyethylene ether group is usually heterogeneous, due to the polymerization process from ethylene glycol (Helenius et al., 1979). There is usually also some heterogeneity in the alkyl group (Helenius et al., 1979). Homogeneous detergents of this type are also available.

The detergent used for solubilization of hormone-sensitive lipase (see below) was a heterogenous alkylpolyoxyethylene ether,  $C_{13}E_{12}$  (II, Belfrage et al., 1977). This detergent has also been used for initial purification steps (II, Belfrage et al. 1977). However, in finally purified enzyme preparations a homogeneous detergent,  $C_{12}E_8$ , has been used (II). The main reason for this was that characterization of the enzyme, especially measurement of detergent binding and properties that are dependent on detergent binding, required the use of a well defined detergent.

Yet another detergent has been used in this investigation, namely  $C_8E_6$  (I). This detergent was less heterogeneous than the usual commercially available detergents, but not homogeneous (I). It was used for the so-called affinity chromatography of hormone-sensitive lipase on triacylglycerol-containing Ultrogel AcA-34 (II; see below), since it allowed the controlled interaction of the lipase with the triacylglycerol containing matrix. In the presence of e.g.  $C_{13}E_{12}$ , the Ultrogel AcA-34 column could be used for molecular exclusion chromatography (Fredrikson et al., 1981). The  $C_8E_6$  was available from commercial sources, in a heterogenous form, but this was not satisfactory for the Ultrogel AcA 34-chromatography and the detergent therefore had to be synthesized (I). This synthesis was performed by a slight modification of a previously published method (Corkill et al., 1961), by vacuum distillation followed by coupling to octylbromide and another distillation. This procedure made the polyoxyethylene moiety much less heterogeneous than with conventional condensation procedures. One problem, however, was encountered when coupling of prefractionated polyoxyethylene was used, namely the formation of diethers, i.e. the coupling of alkylgroups to both ends of the polyoxyethylene group. These diethers were found to inhibit hormone-sensitive lipase activity, and they had to be removed from the detergent preparations (by solvent extraction) before use.

C<sub>8</sub>E<sub>6</sub> has also been used for other purposes, e.g. in attempts to reconstitute hormone-sensitive lipase into phospholipid vesicles (C. Holm, G. Fredrikson and P. Belfrage, unpublished). The advantage of C<sub>8</sub>E<sub>6</sub> in this case is that it has a high critical micellar concentration (cmc) (Corkill et al., 1961), i.e. it can be removed by dialysis. C<sub>13</sub>E<sub>12</sub> and C<sub>12</sub>E<sub>8</sub>, due to their low respective cmc values (Borgström, 1976; Meguro et al., 1981), cannot be removed by dialysis in any reasonable time.

Work with detergents sometimes requires the measurement of concentrations of the detergents. This is not an easy task, since chemical analysis is rather complicated (see e.g. Nozawa and Ohnuma, 1980; Whitmore and Wheeler, 1980). In order to simplify measurement of detergent concentration, or simply to be able to trace detergent in an experiment, two <sup>14</sup>C-labelled detergents were also synthesized (I). They were synthesized by essentially the same procedure as the unlabelled C<sub>8</sub>E<sub>6</sub> (I), except that the alkyl moiety was a labelled carboxylic acid that had to be reduced before coupling to the prefractionated polyoxyethylene (I). The [<sup>14</sup>C]C<sub>8</sub>E<sub>6</sub> was closely similar to unlabelled C<sub>8</sub>E<sub>6</sub>, but [<sup>14</sup>C]C<sub>12</sub>E<sub>8</sub> was more heterogeneous than the unlabelled one. Its properties (cmc and micelle size) were similar, however, and it could be further purified to a virtually homogeneous species, if necessary. These two detergents were chosen since one had a high cmc (C<sub>8</sub>E<sub>6</sub>) and one had a low (C<sub>12</sub>E<sub>8</sub>). The high specific activity of the labelled detergents made possible their use in trace quantities, so they should not change the properties of the unlabelled detergent appreciably. This means that they could also be used to trace other, similar, detergents. [<sup>14</sup>C]C<sub>12</sub>E<sub>8</sub> was thus used as a tracer for C<sub>13</sub>E<sub>12</sub> on column chromatographies (II, Belfrage et al., 1979), and both have proved to be valuable tools in the work with hormone-sensitive lipase.



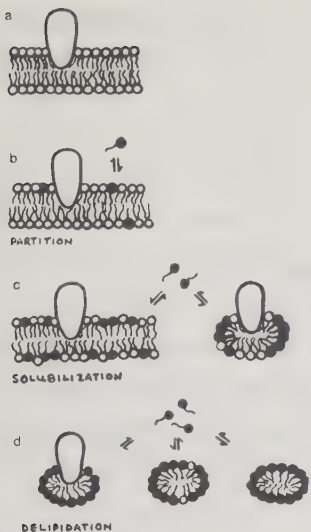


Fig. 2. Different stages in the solubilization of a membrane protein.

- a) Membrane protein embedded in the phospholipid (open circles) bilayer
- b) Addition of detergent (filled circles) in a low concentration leads to partition of the detergent between the membrane and the aqueous phase.
- c) Further addition of detergent solubilizes the protein in mixed detergent/lipid micelles.
- d) At high concentration of detergent the protein becomes delipidated.

Reproduced with the kind permission of Professor Ari Helenius.

Solubilization of hormone-sensitive lipase in detergent was a prerequisite for its purification (cf. Fig. 2). The lipase was first solubilized by Belfrage et al. (1977), but the process of the solubilization had to be further developed in order to achieve complete and reproducible solubilization of the lipase (II; Fredrikson et al., 1981). The detergent concentration used (6.5% w/v) was required to obtain at least 5 detergent micelles/protein molecule (cf. Helenius et al., 1979). Sucrose was found to improve the solubilization and a high salt concentration was also advantageous, but was restricted by the conditions required for successful gradient sievorptive chromatography following the solubilization (II). Sonication was used since it made the process quicker, the conventional 40°C overnight treatment was not enough, and storage for longer time periods resulted in large losses of enzyme activity (partly due to pH-instability). Centrifugation of the solubilized preparation removed remaining insoluble material (and titanium from the sonifier tip) that could otherwise cause problems during the column chromatography. Since the publication of paper II the process has been slightly modi-

fied, so that water is overlaid before the centrifugation, and some remaining unsolubilized neutral lipids are thus easily removed after floatation.

## PURIFICATION

Hormone-sensitive lipase from rat adipose tissue was partially purified, and first identified, in the brief report 1977 by Belfrage et al. The purification procedure could not, however, be used for an extensive purification of the lipase. The reason for this was mainly the low yield of active enzyme in the preparation, in turn a result of irreversible aggregation during the ion exchange chromatography (cf. below). The yield, reported to be about 10% (Belfrage et al., 1977), was probably in reality much lower, due to difficulties in obtaining correct estimates of the enzyme activity in the presence of endogenous lipids or detergent (Strålfors, 1983).

An entirely new purification protocol was therefore developed (Fig. 3). The first steps of the purification, homogenization, centrifugation at 100,000xg to remove floating lipid, and precipitation at pH 5.2 were the same as had been worked out by Huttunen et al. and proved very useful (Huttunen et al., 1970b). In order to enable preparation at a large scale, a method was worked out to store adipose tissue frozen at -80°C. This demanded the use of protease inhibitors (which had not been used with the fresh tissue), since the enzyme activity was otherwise rapidly lost. The commonly used DFP could not be employed, since it also inhibits hormone-sensitive lipase activity (Vaughan et al., 1964; II). Peptide protease inhibitors (leupeptin and pepstatin) were found to protect the enzyme during the fractionation procedure.

A problem in the work of Belfrage et al. (1977) was that the enzyme activity was inhibited by the detergent. It was therefore very difficult to determine the yield of the separation steps. However, if the enzyme fractions were diluted sufficiently before assay of enzyme activity, there was no appreciable inhibition (II). The detergent had to be diluted to a concentration at, or below, its cmc depending on the substrate.

### Purification of Hormone-Sensitive Lipase

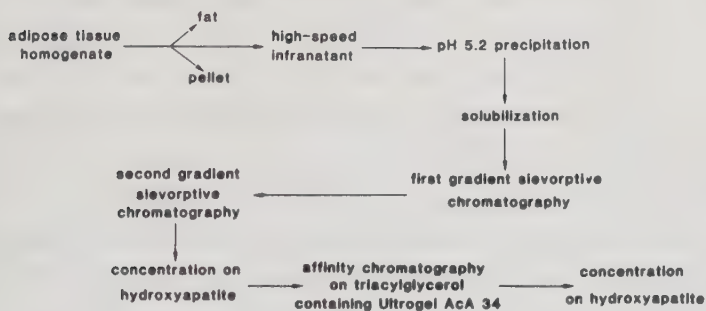


Fig. 3. Outline of the purification protocol for hormone-sensitive lipase.

The first step in the 1977 purification by Belfrage et al. was a conventional ion-exchange chromatography. The enzyme, however, was obtained in a very low yield. It is not uncommon among membrane proteins that ion-exchange chromatography is not suitable for their purification (Hjertén et al., 1981). This is probably due to aggregation of the protein when it is concentrated upon binding to the matrix. An alternative to conventional ion-exchange chromatography, so-called gradient sievortive chromatography (Kirkegaard, 1976) was therefore used (II). The gel matrix used combines the properties of an ion-exchange matrix with molecular sieving properties, enabling proteins to interact electrostatically without being bound to the matrix and concentrated (Kirkegaard, 1976). The yield of enzyme activity was

considerably higher when this method was employed. The first QAE-Sephadex chromatography was very important in the purification from several points of view. Lipids and the surplus of detergent from the solubilization were removed in the void volume, as was monoacylglycerol lipase and all more basic proteins. Lipoprotein lipase was removed to approx. 90% (it had to be traced by  $^{125}\text{I}$ -labelled enzyme, since its activity could not be restored after detergent solubilization). Enzymes involved in the regulation of hormone-sensitive lipase were also removed at this step, e.g. cAMP-dependent protein kinase and protein phosphatases. At the second QAE-Sephadex chromatography further purification was achieved, and remaining lipoprotein lipase was removed. The pH of the QAE-Sephadex columns had to be kept high in order to obtain separation from unretained proteins (pH 7.97 at 10°C), but hormone-sensitive lipase is unstable at pH other than neutral. The column eluate therefore had to be immediately neutralized, and at the same time glycerol was added to a final concentration of 50% (w/v) to improve the stability of the enzyme. By two successive gradient sievortptive chromatographies the enzyme was 150-fold purified from tissue homogenate (II; Table 1). Since this preparation contained no other lipolytic enzymes (monoacylglycerol lipase, lipoprotein lipase, or lysosomal acid lipase) and no enzymes involved in the regulation of hormone-sensitive lipase (cAMP-dependent protein kinase or protein phosphatases) it was very useful for studies of the enzyme activity and its regulation (see below and Strålfors, 1983; Olsson et al., 1984).

The final purification step was an affinity chromatography, utilizing triacylglycerol included in an Ultrogel AcA 34 resin (II). The triacylglycerol remained from the manufacturing procedure, when soybean oil was used in the bead-polymerization of the gel matrix. When the column was equilibrated and eluted in a buffer containing  $\text{C}_8\text{E}_6$ , i.e. a detergent with a high cmc and

when the sample was loaded in a high concentration of phosphate, the enzyme was retained and eluted close to the total column volume. The degree of purification was high, 15-fold (II; Table 1).

After the second QAE-Sephadex and the affinity chromatography steps the enzyme was concentrated on hydroxylapatite (II). In contrast to ultrafiltration, this step enabled concentration of the enzyme without concomitant concentration of the detergent. Another advantage with this procedure was that the detergent could easily be exchanged (cf. Belfrage et al., 1979).

A further development of the purification of hormone-sensitive lipase has taken place since the publication of paper II. The purification has been scaled up, so that adipose tissue from 500-600 rats is used for one purification. The purification steps are also being modified and high-performance liquid chromatography is being developed to prepare the lipase (S. Nilsson, unpublished).

Table 1  
Purification of hormone-sensitive lipase from rat adipose tissue

Enzyme was purified from 220 g of fat pads. Enzyme activity was measured with monooleoylmonooleylglycerol as substrate, and one unit is equivalent to 1  $\mu$ mol of fatty acid produced per min (37°C).

| Purification step                            | Total activity<br>units | Specific activity<br>units/mg<br>protein | Recovery<br>% | Purification<br>-fold |
|----------------------------------------------|-------------------------|------------------------------------------|---------------|-----------------------|
| 1. Tissue homogenate                         | 160                     | 0.049                                    | 100           | 1.0                   |
| 2. 110,000 x g supernatant                   | 100                     | 0.059                                    | 62            | 1.2                   |
| 3. pH 5.2 precipitate                        | 93                      | 0.22                                     | 58            | 4.5                   |
| 4. Solubilized pH 5.2 precipitate            | 88                      | 0.21                                     | 55            | -                     |
| 5. First gradient sievortive chromatography  | 44                      | 3.1                                      | 28            | 63                    |
| 6. Second gradient sievortive chromatography | 23                      | 7.4                                      | 14            | 150                   |
| 7. Concentration on hydroxapatite            | 14                      | 7.2                                      | 8.8           | -                     |
| 8. Affinity chromatography                   | 7.2                     | 100                                      | 4.5           | 2000                  |

Modified from paper II.



### Identification of the enzyme protein

The purified enzyme was identified as the hormone-sensitive lipase by several criteria (II): it had a neutral pH-optimum, no other lipase activity (except monoacylglycerol lipase and lipo-protein lipase) separated during the purification, it had the inhibition properties of hormone-sensitive lipase and it could be activated by cAMP-dependent protein kinase.

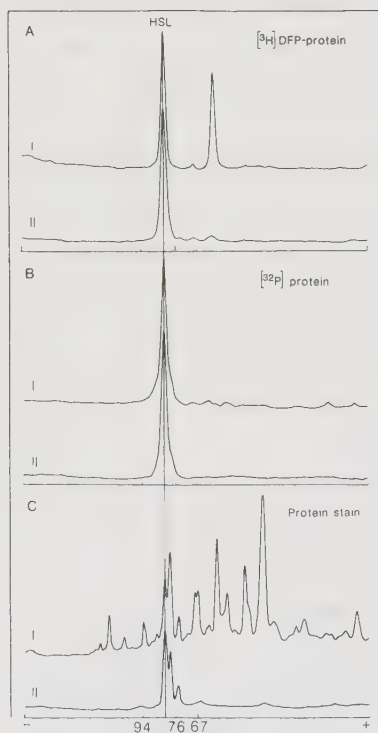


Fig. 4. Scanning densitograms from SDS-PAGE of hormone-sensitive lipase at different purification steps. The enzyme preparation was from before (I) or after (II) the affinity chromatography step. A. Autoradiograph of [<sup>3</sup>H] DFP-protein; B. Autoradiograph of [<sup>32</sup>P] protein and C. Coomassie Blue stain. Modified from paper II.

The identification of hormone-sensitive lipase with the  $M_r=84000$  polypeptide was established since this polypeptide was enriched over several separation steps, in the final enzyme preparation only this polypeptide was labelled with [<sup>3</sup>H] DFP (an inhibitor of hormone-sensitive lipase activity), and the  $M_r=84000$

polypeptide was selectively phosphorylated after incubation with the catalytic subunit of cAMP-dependent protein kinase and [ $\gamma$ - $^{32}\text{P}$ ]ATP (Fig. 4).

The identification by Belfrage et al. (1977) was thus established. The difference in  $M_r$  86000 (Belfrage et al., 1977) and 84000 (II) was due to use of different reference proteins.

#### PHYSICOCHEMICAL PROPERTIES

Only few of the physicochemical properties of hormone-sensitive lipase have yet been examined. Such investigations have been seriously hampered by the limited availability of lipase (approx. 20  $\mu\text{g}$  enzyme protein could be obtained from 200 rats). They have, with few exceptions, been performed with the approx. 4% pure enzyme preparation, obtained after the second gradient sievorpative chromatography, due to the limited availability of the highly purified enzyme, and to the higher concentration of the enzyme that could be obtained in the former preparation. The enzyme was usually traced by its activity. Since hormone-sensitive lipase is an enzyme regulated by phosphorylation-dephosphorylation (cf. Belfrage et al., 1984; Strålfors and Belfrage, 1984) it is also important to know the state of phosphorylation of the enzyme preparation used. In the present investigation the enzyme was used as purified from the tissue homogenate. The regulatory phosphorylation site was not appreciably phosphorylated (Strålfors and Belfrage, 1983). The so-called basal site (phosphorylated by a cAMP-independent process and not directly affecting the enzyme activity) was presumably not phosphorylated, since the rate of dephosphorylation was rapid during the homogenization of the tissue (Strålfors et al., 1984).

The pI of hormone-sensitive lipase was found to be 6.7-6.8 at 40°C by isoelectric focusing (Belfrage et al., 1977; II). There is probably a species difference in the pI of the enzyme, the pig adipose tissue enzyme having a slightly lower pI, as judged by its behaviour on the QAE-Sephadex chromatographies (cf. IV). The hen enzyme, by the same criteria, has a substantially lower pI (E. Degerman, G. Fredrikson and P. Belfrage, unpublished).

The  $M_r$  of the enzyme subunit polypeptide on SDS-PAGE was found to be 83,600  $\pm$  490 (mean  $\pm$  S.D., n=6) (reference proteins:  $\beta$ -galactosidase, 116,000; glycogen phosphorylase, 97,400; transferrin, 76,700; bovine serum albumin, 68,000; catalase, 58,500; ovalbumin, 43,000). The free electrophoretic mobility in SDS was found, by a Ferguson-plot (Banker and Cotman, 1972), to be the same for hormone-sensitive lipase as for the reference proteins at 6.5, 7, 7.5 and 8% gelconcentration, using the SDS-PAGE system developed by Laemmli (1970). When the SDS-PAGE was run in the absence of mercaptoethanol the mobility of the enzyme subunit did not change, but the band was blurred (G. Fredrikson, unpublished).

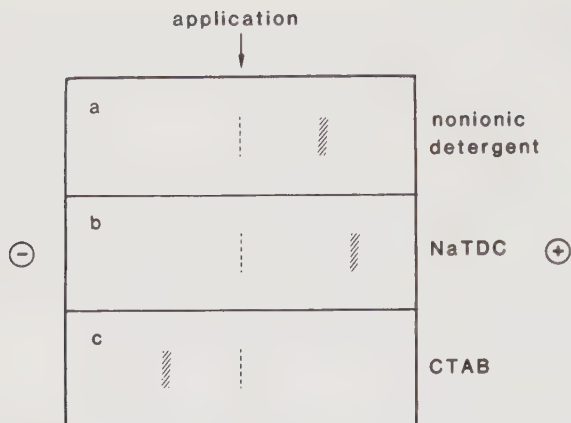
Gel chromatography of hormone-sensitive lipase in the presence of detergents gave an app. molecular weight of 161,000  $\pm$  18,000 (mean  $\pm$  S.D., n=8) on Sephacryl S-300 (reference proteins: IgG, 160,000; bovine serum albumin, 68,000; ovalbumin 43,000; myoglobin 17,000). Also on Sephadex G-200 the app. molecular weight was the same as for IgG (Belfrage et al., 1977). The Stoke's radius was approx. 55Å (Belfrage et al., 1977); the lower Stoke's radius reported in paper II was due to different values given in the reference literature for the standard proteins. In the determinations above the detergent used ( $C_{13}E_{12}$ ) had a micelle size of 98,000 (I). However, the app. molecular weight was found to be the same (162,000  $\pm$  20,000, mean  $\pm$  range n=3) when  $C_8E_6$ , with a micelle size of 17,000 (I), was used.

Ultracentrifugation in sucrose gradients gave a relative sedimentation coefficient of  $4.5 \pm 0.3$  S (mean  $\pm$  S.D.,  $n=9$ , IgG (6.6 S) was used as reference) when measured in  $C_{13}E_{12}$  using essentially the procedure of Martin and Ames (1961). Determination of molecular weight from the data above, demands knowledge of the partial specific volume. Any binding of detergent to the protein molecule changes the partial specific volume. However, the limited availability of enzyme protein has not yet made possible the measurement of detergent binding to the protein.

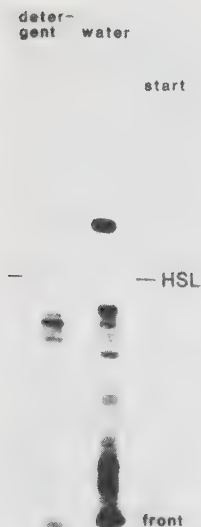
That the enzyme protein indeed interacted with detergent was demonstrated by charge-shift electrophoresis (Helenius and Simons, 1977). The enzyme protein was shown to shift its mobility on electrophoresis, depending on the charge of the detergent present (Fig. 5; G. Fredrikson, unpublished).

Such charge-shifts have previously been shown for intrinsic membrane proteins (Helenius and Simons, 1977), and for other detergent-binding, amphiphilic proteins (lipoprotein lipase, Bengtsson and Olivecrona, 1979a; monoacylglycerol lipase, H. Tornqvist, personal communication). Phase separation in Triton X-114 (Bordier, 1981) was also used to study detergent binding. This detergent has the unique property that it precipitates out of solution at temperatures that are not usually denaturing to proteins. Membrane proteins precipitate with the detergent whereas water-soluble proteins do not (Bordier, 1981). Hormone-sensitive lipase was found to partition between the two phases, with a preference for the detergent phase (Fig. 6; G. Fredrikson, unpublished).

In the latter two experiments the enzyme could not be traced by its activity since it was completely inhibited by the detergents and the separation procedures. Phosphorylation with



**Fig. 5.** Charge shift electrophoresis of hormone-sensitive lipase. Combined drawing of autoradiographs of dried agarose (1% (w/v)) gels after electrophoresis of  $^{32}\text{P}$ -hormone-sensitive lipase in 50 mM glycine-NaOH, pH 9.0, 0.1 M NaCl, 1 mM dithioerythritol and a) 0.2% (w/v)  $\text{C}_{13}\text{E}_{12}$  b) 0.2% (w/v)  $\text{C}_{13}\text{E}_{12}$  and 0.25% (w/v) sodium taurodeoxycholate and c) 0.2% (w/v)  $\text{C}_{13}\text{E}_{12}$  and 0.05% (w/v) cetyltrimethylammonium bromide for 3 h at 4.5 V/cm at room temperature. That the phosphorylated band on the gel corresponded to hormone-sensitive lipase was confirmed by SDS-PAGE of material eluted from a fractionated gel that had been run under the same conditions.



**Fig. 6.** Distribution of  $^{32}\text{P}$ -hormone-sensitive lipase between water and Triton X-114. Hormone-sensitive lipase (HSL) in 5 mM imidazol-HCl, pH 7.2, 50 mM NaCl, 10% (w/v) glycerol, 1 mM dithioerythritol, 0.5% (w/v) Triton X-114 was incubated at 30°C for 3 min and the detergent phase was separated by centrifugation. The procedure was repeated once. The detergent and water phases were analyzed by autoradiography of samples separated by SDS-PAGE.



[ $\gamma$ - $^{32}\text{P}$ ]ATP and the catalytic subunit of cAMP-dependent protein kinase was used to trace the enzyme. Phosphorylation has been shown not to influence the mobility on SDS-PAGE (II), the elution position on gel chromatography or the movement in a sucrose gradient (G. Fredrikson, unpublished). It would therefore seem plausible that the detergent-binding of non-phosphorylated and phosphorylated hormone-sensitive lipase is the same. No measurement of the amount of detergent bound could be obtained by these methods, however.

The easiest explanation to these results is that the detergent-solubilized hormone-sensitive lipase is a dimer, binding detergent in an amount corresponding to less than a micelle (cf. Le Maire et al., 1983). The result from the gel chromatography could indicate either an enzyme dimer, or a monomer binding approx. one micelle of the detergent. However, if the last explanation was correct one would expect a change of the app. molecular weight when a detergent with a smaller micelle size was used, but this was not the case. The relative sedimentation coefficient would also be expected to be lower than the value found if the enzyme was a monomer complexed with a detergent micelle. However, other explanations of the results cannot be excluded, e.g. it has been shown for pancreatic cholesterol esterase that its higher molecular weight by gelchromatography than by SDS-PAGE was due to a far from spherical shape of the molecule (Guy et al., 1981).

Phosphorylation of the enzyme did not seem to have any influence on the results (G. Fredrikson, unpublished), so the phosphorylation probably does not induce changes in the quaternary structure of the detergent-solubilized enzyme, in contrast to what has been shown for e.g. glycogen phosphorylase.

### Native state of the enzyme

Hormone-sensitive lipase behaves operationally like an intrinsic membrane protein (cf. Helenius and Simons, 1976), in that it requires detergent solubilization and the presence of detergent during purification. This association with lipids would be found if the lipase was localized in adipocyte membranes. The lipid association could, however, also be an artefact of the homogenization procedure, resulting from the interaction of hydrophobic residues in the enzyme molecule with lipids in the tissue (cf. Steinberg, 1976).

It has been proposed that hormone-sensitive lipase is a cytosolic protein (Khoo et al., 1972b), since it was found in the 100,000 x g supernatant. This finding does not exclude the possibility that the lipase could be a membrane protein. Intrinsic membrane proteins sediment at 100,000 x g since they are parts of membrane fragments, which consist mainly of phospholipids (Helenius and Simons, 1975). The lipid-protein aggregates in which hormone-sensitive lipase is found contain also triacylglycerols (Huttunen et al., 1970a; II) and the decreased density of the aggregates prevents a sedimentation at 100,000 x g, which could otherwise have been found.

To examine the intracellular localization of the lipase, other methods must be employed, e.g. immunochemical detection of the lipase on electron microscopic preparates.

### ENZYMOLOGICAL PROPERTIES

The enzyme preparation used for these studies was in most cases the most highly purified preparation, 45-50% pure (II). In some cases where much enzyme was needed, the 4-5% pure enzyme preparation which contained no other lipases (see above and II),

was used instead, but the results were always checked with the purest enzyme. The enzyme preparation used was not phosphorylated on the regulatory site (see above).

### Inhibitors

Hormone-sensitive lipase is inhibited by DFP, NaF and  $\text{HgCl}_2$  (II; Björntorp and Furman, 1962; Hollet and Auditore, 1967; Huttunen et al., 1970b; Vaughan et al., 1964). Besides  $\text{HgCl}_2$  other sulfhydryl group modifiers inactivated the enzyme: N-ethylmaleimide, p-chloromercuribenzoate, 2-nitro-5-thiocyanobenzoate, 5,5'-dithiobis-(2-nitro-benzoate) and dithiopyridine (P. Strålfors, unpublished). Enzyme activity inhibitors were used in the early studies of hormone-sensitive lipase, to characterize the enzyme (Hollet and Auditore, 1967; Huttunen et al., 1970b; Vaughan et al., 1964), and later to differentiate between hormone-sensitive lipase and the other lipases in adipose tissue, lipoprotein lipase and monoacylglycerol lipase (Törnqvist et al. 1978b). The use of the inhibitors, of course, also contribute to the understanding of the mechanism of action of the enzyme. DFP is a well-known serin-esterase inhibitor (Cohen et al., 1967; Kraut, 1977), and the inhibition by low concentrations of DFP indicate a serine residue in the active site of the enzyme (Cohen et al., 1967; Kraut, 1977). Many lipases have been reported to be inhibited by DFP or similar serine esterase inhibitors: pancreatic lipase and lipoprotein lipase by millimolar DFP (Smith and Pownall, 1984; Verger, 1984), bile salt-stimulated lipase and monoacylglycerol lipase by micromolar DFP (Bläckberg and Hernell, 1981; Törnqvist and Belfrage, 1976a). These enzymes have all been suggested to have serine residues in their active sites. There is one major difference, however, in the concentration of DFP needed for inhibition, since lipoprotein lipase and pancreatic lipase needed much higher concentrations for inhibition than the other lipases. The active site serine in pancreatic lipase has been questioned, and it has been suggested that the serine residue

should be involved in the interface recognition site (Verger, 1984). Esterases have an active site that can hydrolyze esters, whereas lipases are dependent on, or stimulated by, a substrate interface (Brockman, 1984; Verger, 1980). Lipases are thus supposed to have both an active site and an interface recognition site (Brockman, 1984; Verger, 1980).

Hormone-sensitive lipase is inhibited by sulfhydryl group modifiers (II and above). It has been suggested that a sulfhydryl group is involved in the interface recognition site of hormone-sensitive lipase (P. Strålfors, unpublished).

Hormone-sensitive lipase is the only lipase that has been reported to be inhibited by NaF. This inhibitor has therefore been important in differentiating between different adipose tissue lipases (Törnqvist et al., 1978b). The mechanism of the effect of NaF is not known. However, the fact that 50% inhibition of the enzyme activity was achieved at different NaF concentrations with different acylglycerol substrates indicates that it is involved in the enzyme-substrate interaction (II). The inhibition is readily reversible. Recently, it has been demonstrated that also pancreatic lipase is inhibited by NaF, with 50% inhibition of the activity toward tributyrin at 2 mM NaF (C. Erlanson-Albertsson, personal communication).

Non-ionic detergents also inhibit the activity of hormone-sensitive lipase (Belfrage et al., 1977; II), presumably by interfering with the enzyme-substrate interaction, as has been found for pancreatic lipase (Borgström, 1976). This inhibition is reversible on dilution, and the effect varies with the substrate, triacylglycerol hydrolysis being most susceptible, whereas the activity towards a monoacylglycerol substrate is present even at quite high concentrations of detergent (III).

Table 2

Properties of hormone-sensitive lipase of rat adipose tissue.

|                                                                               |                                                                                                        |
|-------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------|
| App. subunit molecular weight ( $M_r$ , by SDS-PAGE)                          | 84,000                                                                                                 |
| App. molecular size of detergent-solubilized enzyme (from gel chromatography) | 160,000                                                                                                |
| Stoke's radius                                                                | 55Å                                                                                                    |
| Relative sedimentation coefficient                                            | 4.5S                                                                                                   |
| Isoelectric point (+4°)                                                       | 6.7-6.8                                                                                                |
| Substrate specificity                                                         | long-chain tri-, di- and monoacylglycerol, cholesterol-esters (relative maximal activities 1:10:4:1.5) |
| Positional specificity                                                        | 1(3)-ester > 4 times faster than 2-ester bonds                                                         |
| Specific activity                                                             | 400 $\mu\text{mol fatty acid}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$                                  |
| Molar specific activity                                                       | 600 $\text{mol}\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$                                                 |
| pH-optimum                                                                    | 7.0                                                                                                    |
| Inhibitors (50% inhibition of enzyme activity towards trioleoylglycerol)      | DFP      9 $\mu\text{M}$<br>HgCl <sub>2</sub> 11 $\mu\text{M}$<br>NaF      25 mM                       |

Data from papers II and III; Belfrage et al., 1977; Strålfors and Belfrage, 1983 and unpublished results.

Specific activity

The hormone-sensitive lipase preparation, purified to approx. 50% protein purity, was found to have a specific activity of approx. 100  $\mu\text{mol fatty acid released per min and mg}$  (37°C) towards monooleoylmonooleylglycerol (II), corresponding to a specific activity of approx. 200  $\mu\text{mol per min and mg}$  for the pure enzyme (II). However, using [<sup>3</sup>H]DFP-labelling (only catalytically active enzyme being modified (Cohen et al., 1967; II)) the molar specific activity was calculated to be 600  $\text{mol/s/mol}$  (at 37°C). From this, a specific activity of approx. 400  $\mu\text{mol per min and mg}$  could be calculated. It was assumed that the difference between the two specific activities was due to inactivated enzyme protein present in the preparation (II). This was probably not true. Protein concentration was determined by a modification (Törnqvist and Belfrage, 1976b) of the procedure of Lowry et al. (1951), and was probably overestimated since a determination of protein



concentration by total amino acid analysis gave values about half of those previously obtained (Strålfors and Belfrage, 1983). Hormone-sensitive lipase thus has a specific activity of 400  $\mu\text{mol}$  per min and mg at 37°C (Strålfors and Belfrage, 1983).

This specific activity is in the same order of magnitude as those found for other mammalian lipases e.g. monoacylglycerol lipase (Törnqvist and Belfrage, 1976a), hepatic lipase (Jensen and Bensadoun, 1981; Twu et al., 1984), lipoprotein lipase (Bengtsson and Olivecrona, 1979b, 1980a,b) bile salt stimulated lipase (Bläckberg and Hernell, 1981) and lingual lipase (Field and Scow, 1983). The molar specific activity (calculated from [ $^3\text{H}$ ]DFP-labelling) approached that of bovine milk lipoprotein lipase (Bengtsson and Olivecrona, 1979b; 1980a,b). Pancreatic lipase, however, differs from all other mammalian lipases in having a substantially higher molar specific activity (Patton, 1981).

### Substrate specificity

The substrate specificity of hormone-sensitive lipase differs from that of other mammalian lipases, in that it has a much higher activity toward diacylglycerol than toward triacylglycerol (II). The relative acylglycerol hydrolase activities are 1:10:4 against tri-, 1,2(2,3)-di- and 1(3)-monooleoylglycerol, respectively (II). It is probably not uncommon that diacylglycerol is a better substrate for lipases than triacylglycerol, e.g. this is the case with lipoprotein lipase and pancreatic lipase (Bengtsson and Olivecrona, 1979b, 1980a,b; Brockerhoff and Jensen, 1974) but the 10-fold difference found for hormone-sensitive lipase is unique. However, when the enzyme is phosphorylated, by cAMP-dependent protein kinase and ATP, its activity towards a triacylglycerol substrate in vitro is increased 2-3-fold (II; Strålfors and Belfrage, 1983). The activity towards diacylglycerol, is not increased, or only slightly (Heller et al., 1972; Khoo et al.,

1974; Hans Eriksson, unpublished), and the relation between the acylglycerol hydrolase activities then becomes approx. 1:4:2 (cf. above).

Monoacylglycerols were also substrates for hormone-sensitive lipase (II). The optimal monoacylglycerol substrate was a tri-oleoylglycerol emulsion covered with phospholipids and mono-oleoylglycerol (II; Tornqvist et al., 1978b). Also micellar monooleoylglycerol, prepared by sonication in detergent could be used as a substrate (III). 2-Monooleoylglycerol was hydrolyzed at a rate approx. 1/4 of the rate of 1(3)-monooleoylglycerol hydrolysis (III). Several lipases have been shown to hydrolyze monoacylglycerols (see e.g. Bengtsson and Olivecrona, 1979b; Hernell and Bläckberg, 1982).

Hormone-sensitive lipase hydrolyzes also cholesterol oleate, at a rate approx. equal to the rate of triacylglycerol hydrolysis (II). That all acylglycerol and cholesterol ester substrates were hydrolyzed by the same enzyme (active site) was shown by the copurification of all activities during the purification of hormone-sensitive lipase, and by similar inhibition properties (II). This explains the previously reported cholesterol esterase activity in adipose tissue (Pittman et al. 1975, Khoo et al. 1979). Cholesterol esterase activity has not been reported for pancreatic lipase or lipoprotein lipase. Bile-salt stimulated lipase hydrolyzes cholesterol esters, however, at a rate much lower (1%) than the triacylglycerol hydrolysis rate (Bläckberg et al., 1981). Lysosomal acid lipase also shows activity towards both cholesterol esters and triacylglycerols (Warner et al., 1981). It has been suggested that the neutral cholesterol ester hydrolase is of importance in the hydrolysis of stored cholesterol esters in adipose tissue at starvation (Krause and Hartman, 1984).



hydrolysis (III). In agreement with this, 2-monoacylglycerol was hydrolyzed at a rate about 1/4 of the rate of hydrolysis of 1(3)-monoacylglycerol (III).

Hormone-sensitive lipase thus seems to have a definite preference for the 1 or 3-ester bond of its acylglycerol substrate, though the specificity is not absolute (cf. Fig. 8). Pancreatic lipase and lipoprotein lipase have been reported to have an absolute specificity for the 1(3)-ester bonds (Borgström, 1964; Borgström and Carlsson, 1957; Mattson et al., 1952; Mattson and Beck, 1955; Nilsson-Ehle et al., 1971; Nilsson-Ehle et al., 1973). Monoacylglycerol lipase and bile salt-stimulated lipase lack positional specificity (Hernell and Bläckberg, 1982; Tornqvist et al., 1976a). Lysosomal acid lipase has been reported to, like hormone-sensitive lipase, have a preference, and not an absolute positional specificity (Warner et al., 1981).

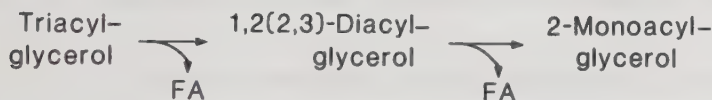


Fig. 8. Postulated main reaction sequence of hormone-sensitive lipase.

#### HORMONE-SENSITIVE LIPASE IN ADIPOSE TISSUE FROM OTHER SPECIES

Rat adipose tissue hormone-sensitive lipase has been purified to a highly purified state and the identity of the enzyme protein established (II). Hormone-sensitive lipase from other species have not been as extensively purified. From several species the crude pH 5.2 precipitate fraction has been prepared (Khoo et al., 1972a; Khoo et al., 1974; Khoo and Steinberg, 1975), and the lipase has been shown to become activated by cAMP-dependent protein kinase and ATP in most species (Khoo and Steinberg, 1975). Swine adipose tissue was used as a source for a further

purification of hormone-sensitive lipase (IV) in an attempt to find a more easily available source for the enzyme than the rat adipose tissue. In view of the discussion on the possible species difference (see below) it was also of interest to study the lipase from another species than the rat. The purification scheme developed for rat adipose tissue was used, with slight modifications (most of them due to a lower pI of the swine enzyme). The purity of the lipase in the final preparation was about 10%, as compared to approx. 50% for the rat enzyme. The enzyme was identified by the selective enrichment of a [ $^3\text{H}$ ] DFP- and  $^{32}\text{P}$ -labelled polypeptide, with an  $M_r=84000$ , having exactly the same mobility on SDS-PAGE as the rat enzyme (II). The two lipases had very similar substrate specificities and inhibition properties (IV). The hormone-sensitive lipases from rat and swine adipose tissue are thus very similar, and the enzyme isolated from mouse differentiated 3T3-cells also seems to be closely similar (Kawamura et al., 1981; Khoo et al., 1981a).

However, hen adipose tissue hormone-sensitive lipase has been partially purified and reported to be identified as an  $M_r=42000$  polypeptide (Berglund et al., 1980; Khoo and Steinberg, 1981; Khoo and Steinberg, 1983), based on the phosphorylation of the major  $M_r=42000$  polypeptide. The properties of this lipase was somewhat different from that of the rat and swine lipases in that it hydrolyzed tri- and diacylglycerols but not monoacylglycerols and cholesterol esters. The most striking difference, however, was the very low specific activity of the lipase, 0.07  $\mu\text{mol}$  fatty acid released per min and mg as compared to 100  $\mu\text{mol}$  per min and mg for the most highly purified preparation of the rat enzyme (II). Other lipases have high specific activities, in the same order of magnitude as the rat enzyme or higher (see above). This fact makes it probable that the  $M_r=42000$  polypeptide, identified as the hen hormone-sensitive lipase, is a contaminant and that the lipase is a much smaller component, not even visible on the



SDS-PAGE gels (see also Belfrage et al., 1984). Recently obtained results of a partial purification of hen hormone-sensitive lipase, where essentially the same procedure was used as for the rat and swine enzymes (II,IV), indicate that the  $M_r=42000$  polypeptide does not copurify with the enzyme activity over the first QAE-Sephadex column fractions (E. Degerman, G. Fredrikson and P. Belfrage, unpublished).

#### FUNCTION OF HORMONE-SENSITIVE LIPASE IN RAT ADIPOSE TISSUE LIPOLYSIS

Hormone-sensitive lipase was originally defined as the triacylglycerol hydrolyzing enzyme, active at neutral pH, that was responsible for the rate-limiting step in adipose tissue lipolysis (Rizack, 1961; Vaughan et al., 1964). Most of the monoacylglycerol hydrolase activity was thought to belong to another enzyme (Gorin and Shafrir, 1964; Heller and Steinberg, 1972; Vaughan et al., 1964). This was confirmed when the monoacylglycerol lipase was purified (Tornqvist and Belfrage, 1976a). However, since purified hormone-sensitive lipase also showed activity toward monoacylglycerols (II), it seemed possible that this enzyme could alone catalyze all the steps in adipose tissue hydrolysis of triacylglycerols. The finding that monoacylglycerols accumulated during incubation of hormone-sensitive lipase with tri- or diacylglycerol (II; Berglund et al., 1980), indicated that this was not the case. The explanation for the accumulation of monoacylglycerols was found to be the positional specificity of hormone-sensitive lipase, leaving 2-monoacylglycerols as a substantial part of the reaction products with subsequent hydrolysis at a relatively low rate (III).

When the action of hormone-sensitive lipase alone, or in combination with monoacylglycerol lipase, on tri- or diacylglycerol substrates was examined, it was found that monoacylglycerol

lipase indeed had an important role in hydrolyzing the monoacylglycerols formed by the action of hormone-sensitive lipase (V). An antiserum against monoacylglycerol lipase was used to inhibit this lipase in a crude adipose tissue preparation. The substantial decrease in glycerol release caused by this demonstrated that monoacylglycerol lipase was essential for the complete hydrolysis of the triacylglycerols to glycerol and fatty acids (V).

The roles of the two lipases in vivo cannot be definitely proven by such experiments. However, it is known that monoacylglycerols do not accumulate in vivo (Arner and Östman, 1974; Scow, 1965; Scow et al., 1965), and it seems reasonable that the functions of the two lipases in vitro are the same in vivo.

Adipose tissue contains mono- and diacylglycerols in a low amount (Arner and Östman, 1974; Scow, 1965; Scow et al., 1965; Vaughan and Steinberg, 1963). The rate-limiting step in lipolysis is the hydrolysis of triacylglycerol. It has sometimes been claimed that diacylglycerols accumulate in adipose tissue when hormone-sensitive lipase activity is stimulated (Scow et al., 1965; Scow, 1965; Arner and Östman, 1974), which would indicate that the hydrolysis of diacylglycerol should become rate-limiting when lipolysis is stimulated by hormones. When hormone-sensitive lipase is activated in vitro the rate of hydrolysis of triacylglycerol is increased more than the rate of hydrolysis of diacylglycerol (see above). However, the rate of triacylglycerol hydrolysis is still considerably lower and the increase in diacylglycerol content probably only reflects a new, higher, steady-state concentration (cf. V and Belfrage et al., 1984).

To summarize: the role of hormone-sensitive lipase in adipose tissue is to catalyze the hydrolysis of triacylglycerols (the rate-limiting step), and of the diacylglycerols (mainly 1,2(2,3)-isomers) produced. Monoacylglycerol lipase will then hydrolyze the monoacylglycerols (mainly 2-isomers) formed (V, Fig. 9).

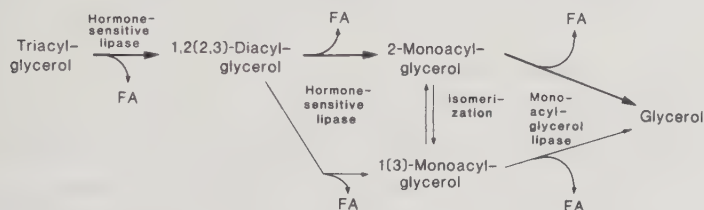


Fig. 9. Postulated reaction sequence in adipose tissue lipolysis, including the roles of hormone-sensitive lipase and monoacylglycerol lipase.

#### HORMONE-SENSITIVE LIPASE - THE SAME ENZYME AS THE ADRENAL CORTEX CHOLESTEROL ESTER HYDROLASE

Adrenal cortex contains a neutral cholesterol ester hydrolase, which has been implicated in the rate-limiting step in steroidogenesis (Boyd and Gorban, 1980). This enzyme was first identified as a  $M_r=41000$  polypeptide, with a specific activity of 0.75 nmol fatty acids released per min and mg protein (Beckett and Boyd, 1977). As was later shown by Cook et al. (1981), the  $M_r=41000$  polypeptide was a major contaminant of the preparation, and the cholesterol ester hydrolase was identified as a  $M_r=84000$  polypeptide, by specific labelling with  $[^3H]$ DFP (Cook et al., 1981). Adrenal cortex has also been reported to contain a neutral triacylglycerol lipase activity (Gorban and Boyd, 1977; Pittman and Steinberg, 1977). The relationship of this lipase activity to the cholesterol ester hydrolase has been discussed (Gorban and Boyd, 1977; Pittman and Steinberg, 1977). Two different lipase activities from bovine adrenal cortex could be resolved, one

probably the same enzyme as the cholesterol ester hydrolase, the other probably the adrenal cortex heparin-releasable lipase (Jansen and de Greef, 1981; Jansen et al., 1980; Lee and Yeaman, 1980; Yeaman et al., 1980). These results were later confirmed (Cook et al., 1981; Cordle et al., 1983; Sonnenborn et al., 1982; Trzeciak et al., 1984).

The neutral lipase/esterase in bovine adrenal cortex exhibited the following properties: similar rates of hydrolysis of cholesterol esters and triacylglycerols; inhibition by micromolar DFP; subunit  $M_r=84000$  by SDS-PAGE (Cook et al., 1981). These properties were similar to the ones reported for hormone-sensitive lipase of rat adipose tissue (II). The cooperative work reported in paper VI was therefore undertaken to directly compare the two enzymes. Several properties of the enzymes were investigated, the two enzymes always run in parallel experiments under identical conditions. (The enzymes were purified separately in the two different laboratories, and brought to our laboratory for the comparative studies.)

Table 3 shows a summary of the properties of the enzyme preparations from the two tissue sources. Due to the presence of monoacylglycerol lipase in the cholesterol ester hydrolase preparation, alkyl analogues were used as substrates instead of acylglycerols: monooleoylmonooleylglycerol instead of dioleoylglycerol and dioleoylmonooleylglycerol instead of trioleoylglycerol, since the products from these substrates can not be hydrolyzed by monoacylglycerol lipase (Fredrikson et al., 1981; Tornqvist et al., 1972; Tornqvist et al., 1978a). Both enzyme preparations were shown to become labelled by [ $^3\text{H}$ ]DFP, both were phosphorylated by [ $\gamma$ - $^{32}\text{P}$ ]ATP and the catalytic subunit of cAMP-dependent protein kinase and the enzymes had identical mobilities on SDS-PAGE (VI). Peptide mapping of [ $^3\text{H}$ ]DFP-labelled enzymes, obtained in gel slices after separation on SDS-PAGE, was carried

out after limited proteolysis and a second SDS-PAGE. The peptide pattern in the lower  $M_r$  region was very similar for the two enzymes (VI).

Table 3

Comparison of the properties of rat adipose tissue hormone-sensitive lipase and bovine adrenal cortex cholesterol ester hydrolase.

|                                                                      | HSL      | CEH      |
|----------------------------------------------------------------------|----------|----------|
| App. molecular weight (SDS-PAGE)                                     | 84000    | 84000    |
| Substrate specificity (relative maximal activities towards TG:CE:DG) | 5:14:100 | 5:10:100 |
| Inhibition of enzyme activity towards CE at                          |          |          |
| 10 $\mu$ M DFP                                                       | 47%      | 48%      |
| 7.5 $\mu$ M HgCl <sub>2</sub>                                        | 46%      | 39%      |
| 30 mM NaF                                                            | 35%      | 34%      |
| Activation of enzyme activity towards TG by                          |          |          |
| ATP-protein kinase                                                   | 48%      | 43%      |
| TG, dioleoylmonooleylglycerol                                        |          |          |
| CE, cholesterol oleate                                               |          |          |
| DG, monooleoylmonooleylglycerol                                      |          |          |

Data from paper VI.

All these similarities between the enzymes in addition to the similar specific activities (II; Cook et al., 1981) and the amphiphilic character of the enzymes (e.g. need for detergent solubilization) makes it very likely that adrenal cortex cholesterol ester hydrolase and adipose tissue hormone-sensitive lipase is the same enzyme (VI). The [<sup>3</sup>H]DFP-peptide patterns (VI), though showing obvious similarities, clearly show that there are also differences between the two proteins. Since they were purified from different species, the differences could of course be due to a species difference, but a tissue difference



could not be excluded. Enzymes from the same species are required to answer this question. The enzymological similarities are obvious, however. Together with the identical subunit sizes, it seems reasonable to state that from a functional point of view, the enzymes are one and the same. The finding that the enzymes are the same in the two tissues means that these two apparently unrelated metabolic systems, lipolysis in adipose tissue and steroid production in adrenal cortex are regulated hormonally by phosphorylation of the same enzyme protein, resulting in different physiological effects depending on the substrate available (Fig. 10).

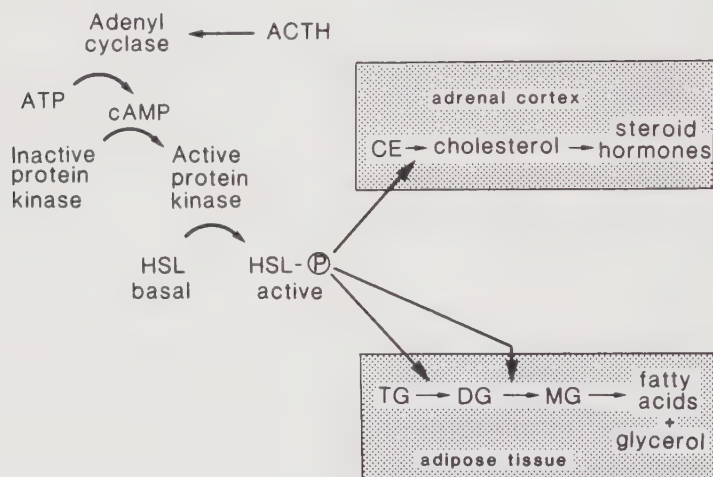


Fig. 10. Outline of the hormonal control of lipolysis and release of free cholesterol in adipose tissue and adrenal cortex, respectively.

Several tissues contain neutral cholesterol ester hydrolases or triacylglycerol lipases, and it is thus possible that the occurrence of hormone-sensitive lipase is more general. A hormonally regulated lipase in heart has been reported (Severson, 1979a; Severson 1979b), and several neutral lipases from e.g. aorta, macrophages, placenta, testis (Chen and Morin, 1971; Durham and McLean Grogan, 1982; Hajjar et al., 1983; Khoo et

al., 1981b; ) could possibly belong to this group of enzymes. However, indications that there are also neutral lipases different from hormone-sensitive lipase have been reported (Khoo et al., 1984). In bovine corpus luteum, the cholesterol ester hydrolase has been shown to be very similar to bovine adrenal cortex cholesterol ester hydrolase, and to the rat adipose tissue lipase (Cook et al., 1983).

Several different steps have been suggested to be rate-limiting in steroidogenesis (Beins et al., 1982; Boyd and Gorban, 1980; Gwynne and Hers, 1980; Verschoor-Klootwyk et al., 1982). Probably, the supply of free cholesterol is important for which step is actually rate-limiting; when this supply is limited, cholesterol ester hydrolase is likely to become the rate-limiting step in hydrolyzing stored cholesterol esters (Strålfors and Belfrage, 1984), whereas ample supply of free cholesterol makes other steps rate-limiting (Löffler and Kuntze, 1984).

## GENERAL DISCUSSION

The purification of rat adipose tissue hormone-sensitive lipase described here, was the first extensive purification of the lipase. The availability of purified lipase has made possible the definite identification of the lipase and also a characterization of some of its properties. This purification was a major breakthrough in the work with hormone-sensitive lipase. During the years from the first description of the lipase in the beginning of the 1960's until 1980 comparatively little new information was gained about the enzyme while, during the last three to four years, there has been a large increase in our knowledge about this metabolically important enzyme (see several recent reviews: Belfrage, 1984; Belfrage et al., 1984; Strålfors and Belfrage, 1984).

When purified lipase was available it enabled a description of the regulation of the activity of the enzyme by reversible phosphorylation (Olsson et al., 1984; Strålfors, 1983; Strålfors et al., 1984). A pure lipase preparation was also a prerequisite for the studies of the regulation of lipolysis in intact adipocytes including the studies on the anti-lipolytic effect of insulin (Belfrage et al., 1980; Nilsson, 1981; Nilsson et al., 1980). The successful purification of hormone-sensitive lipase was the result of development of new methods, e.g. the technique for solubilization of the enzyme in detergent and the adaption of the gradient sievorptive chromatography for use with detergent. Furthermore, the affinity chromatography, which was essential for the high degree of purification, and the hydroxylapatite concentration step, highly important since the detergent could be exchanged and was not concentrated, were the result of adjustments of techniques for use with this amphiphilic enzyme.

The characterization of the lipase is still incomplete. The small amounts of enzyme protein available, the low concentration, the presence of detergent and the fact that no homogeneous preparation has been obtained has hindered these studies. In an effort to solve at least some of these problems swine adipose tissue was used for a purification. However, the concentration of the lipase in this tissue was much lower than in rat adipose tissue, and at the same time the concentration of lipids was higher. This source was therefore not better than rat adipose tissue.

This illustrates that the main problem in the purification of hormone-sensitive lipase is the fact that the lipase, like membrane proteins in general, cannot be concentrated very much since aggregations occur. The limit for the amount of lipase that can be purified is actually the column size and the amounts of buffer that can be handled. Already some improvements of the purifica-

tion method have been made, in that the purification has been scaled up 3-fold and high-performance liquid chromatography is being developed for the final steps and will result in an easier and quicker procedure, with a higher yield of active enzyme.

These new techniques will probably make possible an extension of the characterization of the lipase in the near future. Development of methods to be used in the preparation of antibodies is already going on, and preliminary experiments have been performed to reconstitute the lipase in a phospholipid environment, better resembling the native surroundings in the cell than the detergent complex. Such a reconstituted enzyme preparation is probably a good material for solving the intriguing problem of the mechanism of activation of the enzyme. It is also likely that short sequences of the lipase around the phosphorylated sites and around the active site will be known in the near future. And, thereafter, entirely different methods, like cloning of DNA, might possibly make more enzyme available thus eliminating the current problem with the limited amounts of pure hormone-sensitive lipase.

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## REFERENCES

- Arner, P. and Östman, J. (1974) Mono- and diacylglycerols in human adipose tissue. Biochim. Biophys. Acta 369, 209-221.
- Banker, G.A. and Cotman, C.W. (1972) Measurement of free electrophoretic mobility and retardation coefficient of protein-sodium dodecylsulfate complexes by gel electrophoresis. A method to validate molecular weight estimates. J. Biol. Chem. 247, 5856-5861.
- Beckett, G.J. and Boyd, G.S. (1977) Purification and control of bovine adrenal cortical cholesterol ester hydrolase and evidence for the activation of the enzyme by a phosphorylation. Eur. J. Biochem. 72, 223-233.
- Beins, D.M., Vining, R. and Balasubramaniam, S. (1982) Regulation of neutral cholesterol esterase and acyl-CoA: cholesterol acyltransferase in the rat adrenal gland. Biochem. J. 202, 631-637.
- Belfrage, P. (1984) Hormonal control of lipid degradation. In A. Cryer and R.L.R. Van (eds.), New perspectives in adipose tissue structure, function and development. Butterworths, London. In press.
- Belfrage, P., Fredrikson, G., Nilsson, N.Ö. and Strålfors, P. (1979) Hormone-sensitive lipase of rat adipose tissue. INSERM Colloq. 87, 161-178.
- Belfrage, P., Fredrikson, G., Nilsson, N.Ö. and Strålfors, P. (1980) Regulation of adipose tissue lipolysis: phosphorylation of hormone-sensitive lipase in intact rat adipocytes. FEBS Lett. 111, 120-124.
- Belfrage, P., Fredrikson, G., Strålfors, P. and Tornqvist, H. (1984) Adipose tissue lipases. In B. Borgström and H.L. Brockman (eds.) Lipases, Elsevier/North Holland, Amsterdam, New York, pp. 365-416.

- Belfrage, P., Jergil, B., Strålfors, P. and Tornqvist, H. (1977) Hormone-sensitive lipase of rat adipose tissue: identification and some properties of the enzyme protein. FEBS Lett. 75, 259-264.
- Bengtsson, G. and Olivecrona, T. (1979a) Binding of deoxycholate to lipoprotein lipase. Biochim. Biophys. Acta 575, 471-474.
- Bengtsson, G. and Olivecrona, T. (1979b) Apolipoprotein CII enhances hydrolysis of monoglycerides by lipoprotein lipase, but the effect is abolished by fatty acids. FEBS Lett. 106, 345-348.
- Bengtsson, G. and Olivecrona, T. (1980a) Lipoprotein lipase: some effects of activator proteins. Eur. J. Biochem. 106, 549-555.
- Bengtsson, G. and Olivecrona, T. (1980b) Lipoprotein lipase. Mechanism of product inhibition. Eur. J. Biochem. 106, 557-562.
- Berglund, L., Khoo, J.C., Jensen, D. and Steinberg, D. (1980) Resolution of hormone-sensitive triglyceride/diglyceride lipase from monoglyceride lipase of chicken adipose tissue. J. Biol. Chem. 255, 5420-5428.
- Björntorp, P. and Furman, R.H. (1962) Lipolytic activity in rat epididymal fat pads. Am. J. Physiol. 203, 316-322.
- Bläckberg, L. and Hernell, O. (1981) The bile-salt-stimulated lipase in human milk. Purification and characterization. Eur. J. Biochem. 116, 221-225.
- Bläckberg, L., Lombardo, D., Hernell, O., Guy, O. and Olivecrona, T. (1981) Bile salt-stimulated lipase in human milk and carboxylester hydrolase in pancreatic juice. Are they identical enzymes? FEBS Lett. 136, 284-288.
- Bordier, C. (1981) Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. 256, 1604-1607.
- Borgström, B. (1964) Influence of bile salts, pH, and time on the action of pancreatic lipase; physiological implications. J. Lipid Res. 5, 522-531.

- Borgström, B. (1976) Binding of pancreatic colipase to interfaces; effects of detergents. FEBS Lett. 71, 201-204.
- Borgström, B. and Carlson, L.A. (1957) On the mechanism of the lipolytic action of the lipaemia-clearing factor. Biochim. Biophys. Acta 24, 638-639.
- Boyd, G.S. and Gorban, A.M.S. (1980) Protein phosphorylation and steroidogenesis. In P. Cohen (ed.), Molecular Aspects of Cellular Regulation. Vol. 1. Newly Discovered systems of Enzyme Regulation by Reversible Phosphorylation. Elsevier/North-Holland, Amsterdam, New York, pp. 95-134.
- Brockerhoff, H. and Jensen, R.G. (1974) Lipolytic Enzymes. Academic Press, New York, pp. 34-90.
- Brockman, H.L. (1984) General features of lipolysis: reaction scheme, interfacial structure and experimental approaches. In B. Borgström and H. L. Brockman (eds.) Lipases, Elsevier/North Holland, Amsterdam, New York, pp. 3-46.
- Chen, L. and Morin R. (1971) Purification of a human placental cholesteryl ester hydrolase. Biochim. Biophys. Acta 231, 194-197.
- Cohen, J.A., Oosterbaan, R.A. and Berends, F. (1967) Organophosphorus compounds. Methods Enzymol. 11, 686-702.
- Cook, K.G., Lee, F.-T. and Yeaman, S.J. (1981) Hormone-sensitive cholesterol ester hydrolase of bovine adrenal cortex. Identification of the enzyme protein. FEBS Lett. 132, 10-13.
- Cook, K.G., Colbran, R.J., Snee, J. and Yeaman, S.J. (1983) Cytosolic cholesterol ester hydrolase from bovine corpus luteum: its purification, identification and relationship to hormone-sensitive lipase. Biochim. Biophys. Acta 752, 46-53.
- Cordle, S.R., Yeaman, S.J. and Clegg, R.A. (1983) Salt-resistant (hepatic) lipase. Evidence for its presence in bovine liver and adrenal cortex. Biochim. Biophys. Acta 753, 213-219.
- Corkill, J.M., Goodman, J.F. and Ottewill, R.H. (1961) Micellization of homogeneous non-ionic detergents. Trans. Faraday Soc. 57, 1627-1636.

- Durham, III., L.A. and McLean Grogan, W. (1982) Temperature sensitivity of cholesterol ester hydrolases in the rat testis. Lipids 17, 970-975.
- Field, R.B. and Scow, R.O. (1983) Purification and characterization of rat lingual lipase. J. Biol. Chem. 258, 14563-14569.
- Fredrikson, G., Strålfors, P., Nilsson, N.Ö. and Belfrage, P. (1981) Hormone-sensitive lipase from adipose tissue of rat. Methods Enzymol. 71, 636-646.
- Fowler, S.D. and Brown, W.J. (1984) Lysosomal acid lipase. In B. Borgström and H.L. Brockman (eds.) Lipases, Elsevier/North Holland, Amsterdam, New York, pp. 329-364.
- Gorban, A.M.S. and Boyd, G.S. (1977) ACTH activation of cytosol triglyceride hydrolase in the adrenal of the rat. FEBS Lett. 79, 54-58.
- Gorin, E. and Shafrir, E. (1964) Lipolytic activity in adipose tissue homogenate toward tri-, di-, and monoglyceride substrates. Biochim. Biophys. Acta 84, 24-34.
- Guy, O., Lombardo, D. and Brahms, J.G. (1981) Structure and conformation of human pancreatic carboxyl-ester hydrolase. Eur. J. Biochem. 117, 457-460.
- Gwynne, J.T. and Hess, B. (1980) The role of high density lipoproteins in rat adrenal cholesterol metabolism and steroidogenesis. J. Biol. Chem. 255, 10875-10883.
- Hajjar, D.P., Minick, C.R. and Fowler, S. (1983) Arterial neutral cholesteryl esterase. A hormone-sensitive enzyme distinct from lysosomal cholesteryl esterase. J. Biol. Chem. 258, 192-198.
- Helenius, A., McCaslin, D.R., Fries, E. and Tanford, C. (1979) Properties of detergents. Methods Enzymol. 61, 734-749.
- Helenius, A. and Simons, K. (1975) Solubilization of membranes by detergents. Biochim. Biophys. Acta 415, 29-79.
- Helenius, A. and Simons, K. (1977) Charge-shift electrophoresis: simple method for distinguishing between amphiphilic and hydrophilic proteins in detergent solution. Proc. Natl. Acad. Sci. U.S.A. 74, 529-532.



- Heller, R.A. and Steinberg, D. (1972) Partial glyceridase activity of a protein-kinase activatable triglyceride lipase from rat adipose tissue. Biochim. Biophys. Acta 270, 65-73.
- Hernell, O. and Bläckberg, L. (1982) Digestion of human milk lipids: physiological significance of sn-2 monoacylglycerol hydrolysis by bile salt-stimulated lipase. Pediat. Res. 16, 882-885.
- Hjertén, J., Pan, H. and Yao, K. (1981) Some general aspects of the difficulties to purify membrane proteins. In H. Peeters (ed.) Peptides of the Biological Fluids. Pergamon Press, pp. 15-25.
- Hollenberg, C.H., Raben, M.S. and Astwood, E.B. (1961) The lipolytic response to corticotropin. Endocrinology 68, 589-598.
- Hollett, C.R. (1964) Adipose tissue: hormonal influence on distribution of lipolytic activity in homogenate fractions. Biochem. Biophys. Res. Commun. 15, 575-580.
- Hollett, C.R. and Auditore, J.V. (1967) Localization and characterization of a lipase in rat adipose tissue. Arch. Biochem. Biophys. 121, 423-436.
- Huttunen, J.K., Aquino, A.A. and Steinberg, D. (1970a) A purified triglyceride lipase, lipoprotein in nature, from rat adipose tissue. Biochim. Biophys. Acta 224, 295-298.
- Huttunen, J.K., Ellingboe, J., Pittman, R.C. and Steinberg, D. (1970b) Partial purification and characterization of hormone-sensitive lipase from rat adipose tissue. Biochim. Biophys. Acta 218, 333-346.
- Jansen, H. and De Greef, W.J. (1981) Heparin-releasable lipase activity of rat adrenals, ovaries and testes. Biochem. J. 196, 739-745.
- Jansen, H., Kalkman, C., Birkenhäger, J.C. and Hülsmann, W.C. (1980) Demonstration of a heparin-releasable liver-lipase-like activity in rat adrenals. FEBS Lett. 112, 30-34.

- Jensen, G.L. and Bensadoun, A. (1981) Purification, stabilization and characterization of rat hepatic triglyceride lipase. Analytical Biochemistry 113, 246-252.
- Kawamura, M., Jensen, D.F., Wancewicz, E.V., Joy, L.L., Khoo, J.C. and Steinberg, D. (1981) Hormone-sensitive lipase in differentiated 3T3-L1 cells and its activation by cyclic AMP-dependent protein kinase. Proc. Natl. Acad. Sci. U.S.A. 78, 732-736.
- Khoo, J.C., Aquino, A.A. and Steinberg, D. (1974) The mechanism of activation of hormone-sensitive lipase in human adipose tissue. J. Clin. Invest. 53, 1124-1131.
- Khoo, J.C., Drevon, C.A. and Steinberg, D. (1979) The hydrolysis of cholesterol esters in plasma lipoproteins by hormone-sensitive cholesterol esterase from adipose tissue. J. Biol. Chem. 254, 1785-1787.
- Khoo, J.C., Fong, W.W. and Steinberg, D. (1972a) Activation of hormone-sensitive lipase from human adipose tissue by cyclic AMP-dependent protein kinase. Biochem. Biophys. Res. Commun. 49, 407-413.
- Khoo, J.C., Jarett, L., Mayer, S.E. and Steinberg, D. (1972b) Subcellular distribution of and epinephrine-induced changes in hormone-sensitive lipase, phosphorylase, and phosphorylase kinase in rat adipocytes. J. Biol. Chem. 247, 4812-4818.
- Khoo, J.C., Kawamura, M., Jensen, P.F., Wancewicz, E.V., Joy, L.L. and Steinberg, D. (1981a) Development of hormone-sensitive lipase in differentiating 3T3-L1 cells: activation and phosphorylation by cAMP-dependent protein kinase. In O.M. Rosen and E.G. Krebs (eds.) Protein Phosphorylation Book A, Cold Spring Harbor Conferences on Cell Proliferation, Vol. 8. Cold Spring Harbor Laboratory, pp. 675-685.
- Khoo, J.C., Mahoney, E.M. and Steinberg, D. (1981b) Neutral cholesterol esterase activity in macrophages and its enhancement by cAMP-dependent protein kinase. J. Biol. Chem. 256, 12659-12661.

- Khoo, J.C. and Steinberg, D. (1974) Reversible protein kinase activation of hormone-sensitive lipase from chicken adipose tissue. J. Lipid Res. 15, 602-610.
- Khoo, J.C. and Steinberg, D. (1975) Hormone-sensitive triglyceride lipase from rat adipose tissue. Methods Enzymol. 35, 181-189.
- Khoo, J.C. and Steinberg, D. (1981) Hormone-sensitive lipase from chicken adipose tissue including the separation and purification of monoglyceride lipase. Methods Enzymol. 71, 627-636.
- Khoo, J.C. and Steinberg, D. (1983) Hormone-sensitive lipase of adipose tissue. In P.D. Boyer (ed.) The Enzymes vol. XVI, Academic Press, New York; pp. 183-204.
- Khoo, J.C., Steinberg, D., Huang, J.J. and Vagelos, P.R. (1976) Triglyceride, diglyceride, monoglyceride and cholesterol ester hydrolases in chicken adipose tissue activated by adenosine 3':5'-monophosphate-dependent protein kinase. J. Biol. Chem. 251, 2882-2890.
- Khoo, J.C., Vance, J.E., Mahoney, E.M., Jensen, D., Wancewicz, E. and Steinberg, D. (1984) Neutral triglyceride lipase in macrophages. Arteriosclerosis 4, 34-40.
- Kirkegaard, L.H. (1976) Sievorptive chromatography - powerful procedures for rapid enzyme purification. In N. Catsimpoolas (ed.) Methods of Protein Separation, Vol. 2, Plenum Press, New York, pp. 279-319.
- Krause, B.R. and Hartman, A.D. (1984) Adipose tissue and cholesterol metabolism. J. Lipid Res. 25, 97-110.
- Kraut, J. (1977) Serine proteases: structure and mechanism of catalysis. Ann. Rev. Biochem. 46, 331-358.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

- Lee, F.-T. and Yeaman, S.J. (1980) The relationship between hormone-sensitive triacylglycerol lipase and cholesterol esterase from swine adipose tissue. Biochem. Soc. Trans. 8, 728-729.
- Le Maire, M., Kwee, S., Andersen, J.P. and Møller, J.V. (1983) Mode of interaction of polyoxyethyleneglycol detergents with membrane proteins. Eur. J. Biochem. 129, 525-532.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.
- Löffler, B.M. and Kunze, H. (1984) Cholesteryl ester hydrolase (EC 3.1.1.13) is not implicated in the regulation of steroid biosynthesis in rat Leydig cells. 16th Meeting of the Federation of European Biochemical Societies. Abstracts p. 178.
- Martin, R.G. and Ames, B.N. (1961) A method for determining the sedimentation behaviour of enzymes: application to protein mixtures. J. Biol. Chem. 236, 1372-1379.
- Mattson, F.H. and Beck, L.W. (1955) The digestion in vitro of triglycerides by pancreatic lipase. J. Biol. Chem. 214, 115-125.
- Mattson, F.H., Benedict, J.H., Martin, J.B. and Beck, L.W. (1952) Intermediates formed during the digestion of triglycerides. J. Nutr. 48, 335-344.
- Meguro, K., Takasawa, Y., Kawahashi, N., Tabata, Y. and Ueno, M. (1981) Micellar properties of a series of octaethyleneglycol-*n*-alkyl-ethers with homogeneous ethylene oxide chain and their temperature dependence. J. Colloid Interface Sci. 83, 50-56.
- Nilsson, N.Ö. (1981) Studies on the short-term regulation of lipolysis in rat adipocytes with special regard to the anti-lipolytic effect of insulin. Thesis, University of Lund, Sweden, ISBN 91-7222-402-9.

- Nilsson, N.Ö., Strålfors, P., Fredrikson, G. and Belfrage, P. (1980) Regulation of adipose tissue lipolysis: effects of noradrenaline and insulin on phosphorylation of hormone-sensitive lipase and on lipolysis in intact rat adipocytes. FEBS Lett. 111, 125-130.
- Nilsson-Ehle, P., Belfrage, P. and Borgström, B. (1971) Purified human lipoprotein lipase: positional specificity. Biochim. Biophys. Acta 248, 114-120.
- Nilsson-Ehle, P., Egelrud, T., Belfrage, P., Olivecrona, T. and Borgström, B. (1973) Positional specificity of purified milk lipoprotein lipase. J. Biol. Chem. 248, 6734-6737.
- Nozawa, A. and Ohnuma, T. (1980) Improved high-performance liquid chromatographic analysis of ethylene oxide condensates by their esterification with 3,5-dinitrobenzoyl chloride. J. Chromatogr. 187, 261-263.
- Olsson, H., Strålfors, P. and Belfrage, P. (1984) Direct evidence for protein phosphatase-catalyzed dephosphorylation/deactivation of hormone-sensitive lipase from adipose tissue. Biochim. Biophys. Acta. In press.
- Olivecrona, T. and Bengtsson, G. (1984) Lipases in Milk. In B. Borgström and H.L. Brockman (eds.) Lipases, Elsevier/North Holland, Amsterdam, New York, pp. 205-261.
- Patton, J.S. (1981) Gastrointestinal lipid digestion. In L.R. Johnson (ed.) Physiology of the Gastrointestinal Tract, Raven Press, New York, pp. 1123-1146.
- Pittman, R.C., Golanty, E. and Steinberg, D. (1972) A second form of hormone-sensitive lipase from rat adipose tissue homogenates. Biochim. Biophys. Acta 270, 81-85.
- Pittman, R.C., Khoo, J.C. and Steinberg, D. (1975) Cholesterol esterase in rat adipose tissue and its activation by cyclic AMP-dependent protein kinase. J. Biol. Chem. 250, 4505-4511.
- Pittman, R.C. and Steinberg, D. (1977) Activatable cholesterol esterase and triacylglycerol lipase activities of rat adrenal and their relationship. Biochim. Biophys. Acta 487, 431-444.



- Rizack, M.A. (1961) An epinephrine-sensitive lipolytic activity in adipose tissue. J. Biol. Chem. 236, 657-662.
- Rizack, M.A. (1964) Activation of an epinephrine-sensitive lipolytic activity from adipose tissue by adenosine 3',5'-phosphate. J. Biol. Chem. 239, 392-395.
- Schwartz, J.P. and Jungas, R.L. (1971) Studies on the hormone-sensitive lipase of adipose tissue. J. Lipid Res. 12, 553-562.
- Scow, R.O. (1965) Perfusion of isolated adipose tissue: FFA release and blood flow in rat parametrial fat body. In A.E. Renold and G.F. Cahill, Jr. (eds.) Handbook of Physiology, Section 5. Williams and Wilkins, Baltimore, pp. 437-453.
- Scow, R.O., Stricker, F.A., Pick, T.Y. and Clary, T.R. (1965) Effect of ACTH on FFA release and diglyceride content in perfused rat adipose tissue. Annals of the New York Academy of Sciences 131, 288-301.
- Severson, D.L. (1979a) Characterization of triglyceride lipase activities in rat heart. J. Mol. Cell. Cardiol. 11, 569-583.
- Severson, D.L. (1979b) Regulation of lipid metabolism in adipose tissue and heart. Can. J. Physiol. Pharmacol. 37, 923-937.
- Smith, L.C. and Pownall, H.J. (1984) Lipoprotein lipase. In B. Borgström and H.L. Brockman (eds.) Lipases, Elsevier/North Holland, Amsterdam, New York, pp. 263-305.
- Sonnenborn, U., Eiteljörge, G., Trzeciak, W.H. and Kunau, W.-H. (1982) Identical catalytic subunit in both molecular forms of hormone-sensitive cholesterol esterase from bovine adrenal cortex. FEBS Lett. 145, 271-276.
- Steinberg, D. (1976) Interconvertible enzymes in adipose tissue regulated by cyclic AMP-dependent protein kinase. Adv. Cyclic Nucleotide Res. 7, 157-198.
- Strand, O., Vaughan, M. and Steinberg, D. (1964) Rat adipose tissue lipases: hormone-sensitive lipase activity against triglycerides compared with activity against lower glycerides. J. Lipid Res. 5, 554-562.

- Strålfors, P. (1983) Regulation of hormone-sensitive lipase through reversible phosphorylation. Role of cyclic AMP-dependent protein kinase. Thesis, University of Lund, Sweden. ISBN 91-7222-627-7.
- Strålfors, P. and Belfrage, P. (1983) Phosphorylation of hormone-sensitive lipase by cyclic AMP-dependent protein kinase. J. Biol. Chem. 258, 15146-15152.
- Strålfors, P. and Belfrage, P. (1984) Reversible phosphorylation of hormone-sensitive lipase/cholesterol ester hydrolase in the hormonal control of adipose tissue lipolysis and of adrenal steroidogenesis. In P. Cohen (ed.) Molecular Aspects of Cellular Regulation. Vol. 3. Recently Discovered Systems of Enzyme Regulation by Reversible Phosphorylation, Part 2. Elsevier/North Holland, Amsterdam, New York, pp. 27-62.
- Strålfors, P., Björgell, P. and Belfrage, P. (1984) Hormonal regulation of hormone-sensitive lipase in intact adipocytes: Identification of phosphorylated sites and effects on the phosphorylation by lipolytic hormones and insulin. Proc. Natl. Acad. Sci. U.S.A. 81. In press.
- Tanford, C. and Reynolds, J.A. (1976) Characterization of membrane proteins in detergent solutions. Biochim. Biophys. Acta 457, 133-170.
- Tornqvist, H. and Belfrage, P. (1976a) Purification and some properties of a monoacylglycerol hydrolyzing enzyme of rat adipose tissue. J. Biol. Chem. 251, 813-819.
- Tornqvist, H. and Belfrage, P. (1976b) Determination of protein in adipose tissue extracts. J. Lipid Res. 17, 542-545.
- Tornqvist, H., Björgell, P., Krabisch, L. and Belfrage, P. (1978a) Monoacylmonoalkylglycerol as a substrate for diacylglycerol hydrolase activity in adipose tissue. J. Lipid Res. 19, 654-656.
- Tornqvist, H., Krabisch, L. and Belfrage, P. (1972) Rapid assay for hormone-sensitive lipase activity of adipose tissue. J. Lipid Res. 13, 424-426.

- Tornqvist, H., Nilsson-Ehle, P. and Belfrage, P. (1978b) Enzymes catalyzing the hydrolysis of long-chain monoacylglycerols in rat adipose tissue. Biochim. Biophys. Acta 530, 474-486.
- Trzeciak, W.H., Sonnenborn, U., Balkow, C. and Kunau, W.-H. (1984) Regulation of steroidogenesis in rat adrenal gland: identification of the bifunctional, hormone-sensitive cholesterol esterase-triacylglycerol-lipase enzyme protein and its discrimination from hormone-insensitive lipases. Mol. Cell. Endocrinol. 35, 131-141.
- Tsai, S.-C., Belfrage, P. and Vaughan, M. (1970) Activation of hormone-sensitive lipase in extracts of adipose tissue. J. Lipid Res. 11, 466-472.
- Twu, J.-S., Garfinkel, A.S. and Schotz, M.C. (1984) Hepatic lipase. Purification and characterization. Biochim. Biophys. Acta 792, 330-337.
- Warner, T.G., Dambach, L.M., Shin, J.H. and O'Brien, J.S. (1981) Purification of the lysosomal acid lipase from human liver and its role in lysosomal lipid hydrolysis. J. Biol. Chem. 256, 2952-2957.
- Vaughan, M., Berger, J.E. and Steinberg, D. (1964) Hormone-sensitive lipase and monoglyceride lipase activities in adipose tissue. J. Biol. Chem. 239, 401-409.
- Vaughan, M. and Steinberg, D. (1963) Effect of hormones on lipolysis and esterification of free fatty acids during incubation of adipose tissue in vitro. J. Lipid Res. 4, 193-199.
- Verger, R. (1980) Enzyme kinetics of lipolysis. Methods Enzymol. 64, 340-392.
- Verger, R. (1984) Pancreatic lipase. In B. Borgström and H.L. Brockman (eds.) Lipases, Elsevier/North Holland, Amsterdam, New York, pp. 83-150.
- Vérine, A., Salers, P., Ronard, M. and Boyer, J. (1976) Analytical-scale isolation of triacylglycerol lipase from human adipose tissue. FEBS Lett. 66, 127-131.

- Verschoor-Klootwyk, A.H., Verschoor, L., Azhar, S. and Reaven, G.M. (1982) Role of exogenous cholesterol in regulation of adrenal steroidogenesis in the rat. J. Biol. Chem. 257, 7666-7671.
- Whitmore, D.A. and Wheeler, K.P. (1980) A simple method for the separation and assay of non-ionic detergents. J. Biochem. Biophys. Methods 2, 133-138.
- Yeaman, S.J., Cook, K.G. and Lee, F.-T. (1980) The relationship between cholesterol ester hydrolase and triacylglycerol hydrolase from bovine adrenal cortex. FEBS Lett. 120, 212-216.

## Synthesis of $^{14}\text{C}$ -labeled alkyl polyoxyethylene detergents

Gudrun Fredrikson, Lennart Krabisch,  
and Per Belfrage

*Department of Physiological Chemistry 4, University of  
Lund, Lund, Sweden*

**Summary** Procedures for the synthesis and purification of two  $^{14}\text{C}$ -labeled alkyl polyoxyethylene detergent preparations are described. One of the preparations consisted mainly of [ $^{14}\text{C}$ ]dodecyl octaoxyethylene ether, the other consisted mainly of [ $^{14}\text{C}$ ]octyl hexaoxyethylene ether, with critical micellar concentrations of approximately 50  $\mu\text{M}$  and 10 mM, respectively. Condensation of alkyl bromides with fractionated polyethyleneglycol resulted in less polydisperse products than conventional ethylene oxide condensation. Highly purified detergent species could easily be obtained from the preparations by thin-layer chromatography. Their use as tracers greatly simplifies determination of detergent concentration during protein solubilization and fractionation.—**Fredrikson, G., L. Krabisch, and P. Belfrage.** Synthesis of  $^{14}\text{C}$ -labeled alkyl polyoxyethylene detergents. *J. Lipid Res.* 1982. **23**: 1246–1248.

**Supplementary key words** detergent purification • adipose tissue lipases

Non-ionic detergents have become invaluable tools for solubilization and fractionation of labile amphiphilic proteins (1, 2). Triton X-100 has been commonly used, but the UV absorbance due to its aromatic group is a

Abbreviations: alkyl polyoxyethylene ethers with the general formula  $\text{C}_n\text{H}_{2n+1}(\text{OCH}_2\text{CH}_2)_x\text{OH}$  are abbreviated  $\text{C}_n\text{E}_x$ .

draw-back, e.g., when continuous monitoring of protein concentration is desired during column fractionations.

This problem is avoided with alkyl polyoxyethylene ether detergents. Several detergents of this type have been used in our laboratory in work with the lipolytic enzymes in adipose tissue (3–6). Determination of these detergents, however, is difficult, unless labeled detergents are used. In this note we describe the synthesis and purification of two  $^{14}\text{C}$ -labeled alkyl polyoxyethylene detergents, one with a low and one with a high critical micellar concentration, and describe some of their properties. The method used for the synthesis, coupling of the alkyl chain to previously fractionated polyethylene glycol, facilitates the further purification of the detergents to homogeneous species. They can then be used also for characterization of membrane proteins in detergent solution, when homogeneous detergents are required, e.g., for studies of detergent binding.

## MATERIALS AND METHODS

### Labeled detergents

The procedure for the synthesis of these detergents is a modification of the method used in a report by Corkill, Goodman, and Ottewill (7). [ $^{14}\text{C}$ ]C<sub>12</sub>E<sub>8</sub> was synthesized as follows. [ $^{14}\text{C}$ ]Lauric acid (The Radiochemical Center, Amersham, Great Britain) (1 mmol, 1.5 mCi) was reduced with 100 mg of LiAlH<sub>4</sub> in 20 ml of dry diethyl ether and boiled with reflux for 2 hr. The resultant [ $^{14}\text{C}$ ]dodecanol (approx. 1 mmol) was dried and 0.3 ml of 48% HBr and 60  $\mu\text{l}$  of H<sub>2</sub>SO<sub>4</sub> were added and the mixture was boiled with reflux for 5 hr. The [ $^{14}\text{C}$ ]dodecanoyl bromide was extracted with diethyl



ether and washed with 5%  $\text{NaHCO}_3$  (w/v). Polyethyleneglycol (400, Merck, West Germany) was distilled and the fractions containing mainly octaethyleneglycol (1.2 mm Hg, approx. 300°C) were collected. The polyethyleneglycol fractions were analyzed by thin-layer chromatography as the monooleyl esters in ethyl acetate-acetic acid-water 140:32:30 (v/v/v). (The esters were prepared as follows: 0.1 mmol polyethyleneglycol was dissolved in 1.5 ml of chloroform. Oleoylchloride (0.15 mmol) was added and the mixture was left at room temperature for 15 min, after which 0.5 ml of methanol was added for another 15 min to esterify any remaining oleoylchloride.) Two ml of distilled polyethyleneglycol was dissolved in 5 ml of dioxane, 160 mg of sodium was added and the mixture was boiled with reflux overnight. The [ $^{14}\text{C}$ ]dodecanoylbromide (dissolved in 5 ml of dioxane) was then added and the reaction was allowed to proceed for 4 hr under the same conditions. The molar excess of polyethylene glycol was approx. 12 times. The reaction mixture was added to 10 ml of 10% ethanol (v/v), washed four times with a total of 12 ml of light petroleum, extracted into 10 ml of chloroform, which was further washed with water, and dried with anhydrous  $\text{Na}_2\text{SO}_4$ . (A small amount of diether, less than 5% of the  $^{14}\text{C}$ -label, was formed, but it was removed by the light petroleum washes.)

The detergent preparation obtained was subjected to preparative thin-layer chromatography on silicic acid (Kieselgel 60G, Merck, West Germany), 0.5 mm thick, in ethyl acetate-acetic acid-water 140:18:16 (v/v/v). To each plate (200 × 200 mm) 75 mg of detergent was added. The bands corresponding to  $\text{C}_{12}\text{E}_7$ ,  $\text{C}_{12}\text{E}_8$ , and  $\text{C}_{12}\text{E}_9$  (unlabeled homogeneous  $\text{C}_{12}\text{E}_5$  and  $\text{C}_{12}\text{E}_8$  (see below) were used as references) were scraped off and the detergent was eluted with 90% aqueous methanol (v/v). After dilution with water to 30% methanol(v/v), the detergent was extracted twice into a half volume of chloroform, which was then dried with anhydrous  $\text{Na}_2\text{SO}_4$ . The yield of labeled detergent was 140 mg with a specific activity of 2.7  $\mu\text{Ci}/\text{mg}$ . To obtain highly purified [ $^{14}\text{C}$ ]  $\text{C}_{12}\text{E}_8$ , the preparative thin-layer chromatography was repeated and only  $\text{C}_{12}\text{E}_8$  was collected.

[ $^{14}\text{C}$ ]  $\text{C}_8\text{E}_6$  was synthesized essentially in the same way: [ $^{14}\text{C}$ ]octanoic acid (Radiochemical Center, Amersham, Great Britain) (4.6 mmol, 1 mCi) was reduced and the [ $^{14}\text{C}$ ]octylbromide was prepared as above. Polyethylene glycol (300, Merck, West Germany) was distilled and fractions containing mainly hexaethyleneglycol (1.2 mm Hg, 210°C) were collected. After condensation and extraction as above the detergent was further purified on charcoal to remove some colored impurities, but it was not subjected to preparative thin-layer chromatography inasmuch as the initial product was less heterogeneous than the [ $^{14}\text{C}$ ]  $\text{C}_{12}\text{E}_8$  preparation. Two

hundred fifty mg of labeled detergent was obtained with a specific radioactivity of 0.5  $\mu\text{Ci}/\text{mg}$ . Both labeled detergents were stored in chloroform under  $\text{N}_2$  at -20°C.

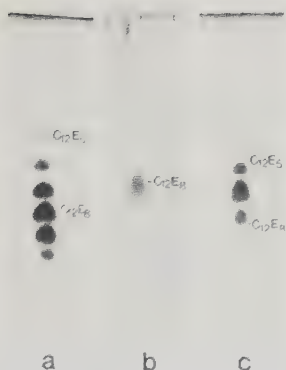
### Unlabeled detergents

Homogeneous  $\text{C}_{12}\text{E}_5$  and  $\text{C}_{12}\text{E}_8$  were obtained from Nikko Chemicals, Tokyo, Japan.  $\text{C}_{13}\text{E}_{12}$  (Berol 058, Berol Kemi, Stenungsund, Sweden) was heterogeneous regarding the carbon chain (54%  $\text{C}_{12}$ , 44%  $\text{C}_{14}$ ) and the polyethyleneglycol moiety.  $\text{C}_8\text{E}_6$  was synthesized essentially as described for [ $^{14}\text{C}$ ]  $\text{C}_8\text{E}_6$ , with the following modifications: octylbromide (Merck, West Germany) was used as one of the reactants and the reaction product was further purified by fractional distillation (0.25 mm Hg, 225–250°C). The final product consisted of  $\text{C}_8\text{E}_6$  (approx. 80%) and  $\text{C}_8\text{E}_5$  only.

### Other methods

Detergent fractions were analyzed by thin-layer chromatography in ethyl acetate-acetic acid-water (140:18:16 (v/v/v).  $\text{C}_{12}\text{E}_5$  and  $\text{C}_{12}\text{E}_8$  were used as references (variation of the alkyl carbon chain length between  $\text{C}_8$  and  $\text{C}_{12}$  did not appreciably effect the  $R_f$ ). The distribution of labeled detergent was determined by liquid scintillation after visualization in iodine vapor, scraping off the spots, addition of 1 ml of 50% methanol (v/v) and 10 ml Instagel (Packard Instrument Co., U.S.A.)-toluene 1:1 (v/v). Critical micellar concentration of the labeled detergents was determined by equilibrium dialysis. Sample, 1 ml, containing 0.5 mM [ $^{14}\text{C}$ ]  $\text{C}_{12}\text{E}_8$  (twice purified by thin-layer chromatography, cf. Fig. 1b) or 30 mM [ $^{14}\text{C}$ ]  $\text{C}_8\text{E}_6$ , was dialyzed against 1 ml of water across a Spectrapor 3 membrane (Spectrum Medical Industries, U.S.A., mol wt cut off approx. 3500) at 25°C  $\pm$  1°C. Aliquots (10  $\mu\text{l}$ ) of the water were taken at intervals of approx. 2 hr, (10  $\mu\text{l}$  was withdrawn from the sample at the same intervals) and counted by liquid scintillation in 10 ml of Instagel-toluene 1:1 (v/v). The concentration of detergent was plotted against time, resulting in a rapid and a slow phase of equilibration which approximated straight lines. The critical micellar concentration was obtained as the detergent concentration at the point of intersection between these two lines.<sup>1</sup> Gel chromatography was performed on Sephacryl S-200 (Pharmacia Fine chemicals, Sweden) with human immunoglobulin G (Kabi, Sweden), bovine albumin, ovalbumin, and myoglobin (Sigma, U.S.A.) as molecular weight references. The column was preequilibrated in a solution of the corresponding unlabeled detergent (0.1%  $\text{C}_{12}\text{E}_8$  (w/v), 0.2%  $\text{C}_{13}\text{E}_{12}$  (w/v), or 0.8%  $\text{C}_8\text{E}_6$  (w/v), respectively).

<sup>1</sup> Lindström, M., and G. Fredrikson. Unpublished results



**Fig. 1.** Thin-layer chromatograms of the labeled detergents. The thin-layer chromatography was performed on silicic acid plates developed in ethyl acetate-acetic acid-water 140:18:16 (v/v/v) with  $C_{12}E_8$  and  $C_{12}E_6$  as references ( $C_{12}E_8$  and  $C_{12}E_6$  in the figure indicate their respective mobilities). The spots were visualized by acid charring. a, [ $^{14}C$ ]  $C_{12}E_8$  after first thin-layer chromatography. The distribution of the labeled detergent (see Methods) was 35%  $C_{12}E_8$ , 20%  $C_{12}E_7$ , and 25%  $C_{12}E_6$ ; b, [ $^{14}C$ ]  $C_{12}E_8$  after second thin-layer chromatography, >85%  $C_{12}E_8$ ; c, [ $^{14}C$ ]  $C_{12}E_8$ -label distribution, 54%  $C_{12}E_8$ , 18%  $C_{12}E_7$ , 17%  $C_{12}E_6$ .

## RESULTS AND DISCUSSION

The somewhat heterogeneous [ $^{14}C$ ]  $C_{12}E_8$  and [ $^{14}C$ ]  $C_8E_6$  preparations (Fig. 1a, c) could be used directly as radioactive tracers in the monitoring of detergent concentration during solubilization and fractionation of amphiphilic proteins. The addition of minute amounts of these labeled preparations should not significantly alter the properties of similar, unlabeled detergents such as  $C_{13}E_{12}$ ,  $C_{12}E_8$ , or  $C_8E_6$ . However, in studies of the binding of detergent by protein, the use of a homogeneous detergent is required. Further purification of the labeled detergent could easily be achieved by repeated thin-layer chromatography (Fig. 1b) but at the expense of the yield. In initial studies, [ $^{14}C$ ] labeled detergent was prepared by conventional ethylene oxide condensation. The labeled detergent so obtained, was, however, considerably more heterogeneous and could not be used for preparation of homogeneous detergent species at any reasonable yield.<sup>2</sup>

The critical micellar concentration of the labeled detergents could only be determined by equilibrium dialysis because of the limited amounts available. The values obtained, 53  $\mu M$  for [ $^{14}C$ ]  $C_{12}E_8$  and 10  $\mu M$  for [ $^{14}C$ ]  $C_8E_6$  at  $25 \pm 1^\circ C$  were of the same magnitude as those reported for homogeneous  $C_{12}E_8$ , 71  $\mu M$  (8) and

$C_8E_6$ , 9.9  $\mu M$  (7); the differences were possibly due to the fact that surface tension measurements were used to determine critical micellar concentration in these reports.

The [ $^{14}C$ ] labeled detergents were used as tracers to determine average micellar molecular weight of the unlabeled alkyl polyoxyethylene detergents  $C_{12}E_n$  (homogeneous),  $C_{13}E_{12}$ , and  $C_8E_6$  (see above) by gel chromatography. Values of 57,000 ( $C_{12}E_n$ ), 98,000 ( $C_{13}E_{12}$ ), and 17,000 ( $C_8E_6$ ) were found as compared with 65,000 ( $C_{12}E_8$ ) and 13,000 (homogeneous  $C_8E_6$ ) reported by others (7, 9). The slightly higher value found for  $C_8E_6$  may be due to the limitations of gel chromatography as a method for determination of micellar size.

The [ $^{14}C$ ] labeled detergents described in this note have proved quite useful in the work with the hormone-sensitive lipase of adipose tissue in this laboratory over a number of years (3-6). They should be equally useful in similar fractionation work with other amphiphilic proteins.

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## REFERENCES

- Helenius, A., and K. Simons. 1975. Solubilization of membranes by detergents. *Biochim. Biophys. Acta.* **415**: 29-79.
- Tanford, C., and J. A. Reynolds. 1976. Characterization of membrane proteins in detergent solution. *Biochim. Biophys. Acta.* **457**: 133-170.
- Tornqvist, H., and P. Belfrage. 1976. Purification and some properties of a monoacylglycerol-hydrolyzing enzyme of rat adipose tissue. *J. Biol. Chem.* **251**: 813-819.
- Fredrikson, G., P. Strålfors, N. Ö. Nilsson, and P. Belfrage. 1981. Hormone-sensitive lipase of rat adipose tissue. Purification and some properties. *J. Biol. Chem.* **256**: 6311-6320.
- Fredrikson, G., P. Strålfors, N. Ö. Nilsson, and P. Belfrage. 1981. Hormone-sensitive lipase from adipose tissue of rat. *Methods Enzymol.* **71**: 636-646.
- Tornqvist, H., and P. Belfrage. 1981. Monoacylglycerol lipase from rat adipose tissue. *Methods Enzymol.* **71**: 646-652.
- Corkill, J. M., J. F. Goodman, and R. H. Ottewill. 1961. Micellization of homogeneous non-ionic detergents. *Trans. Faraday Soc.* **57**: 1627-1636.
- Meguro, K., Y. Takasawa, N. Kawahashi, Y. Tabata, and M. Ueno. 1981. Micellar properties of a series of octa-ethylene glycol-*n*-alkyl ethers with homogeneous ethylene oxide chain and their temperature dependence. *J. Colloid Interface Sci.* **83**: 50-56.
- Helenius, A., D. R. McCaslin, E. Fries, and C. Tanford. 1979. Properties of detergents. *Methods Enzymol.* **56**: 734-749.

<sup>2</sup> Fredrikson, G., L. Krabisch, and P. Belfrage. Unpublished results.



## Hormone-sensitive Lipase of Rat Adipose Tissue

### PURIFICATION AND SOME PROPERTIES\*

Gudrun Fredrikson, Peter Strålfors, Nils Östen Nilsson, and Per Belfrage

From the Department of Physiological Chemistry 4, University of Lund, S-220 07 Lund, Sweden

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Hormone-sensitive lipase from rat adipose tissue was solubilized with nonionic detergent and purified 2,000-fold to 50% protein purity by repeated gradient sievortype chromatography on diethyl-(2-hydroxypropyl)aminoethyl-Sephadex and by affinity chromatography on triacylglycerol-containing polyacrylamide-agarose gel. The identity of the enzyme protein, a polypeptide with  $M_r = 84,000$  by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, was established by its copurification with lipase activity, selective labeling with [ $^3\text{H}$ ]diisopropyl fluorophosphate, and phosphorylation by the catalytic subunit of cAMP-dependent protein kinase from the same tissue and ATP-Mg. There was no indication for a role of a specific lipase kinase analogous to phosphorylase kinase. The phosphorylation enhanced the trioleoylglycerol lipase activity to 150% over that in control when approximately 0.5 mol of phosphate had been incorporated into each mol of its  $M_r = 84,000$  enzyme protein. The purified lipase catalyzed the hydrolysis of emulsified tri-, di-, and monooleoylglycerol and cholesterol oleate at the relative rates of 1:10:4:1.5, with a molar specific activity of 600 mol/s/mol of  $M_r = 84,000$  enzyme protein toward dioleoylglycerol. Its specific activity was more than 1,000-fold higher than that of a lipase recently purified from hen adipose tissue (Berglund, L., Khoo, J. C., Jensen, D., and Steinberg, D. (1980) *J. Biol. Chem.* 255, 5420-5428). It had a pH optimum of 7.0, was inhibited by micromolar diisopropyl fluorophosphate,  $\text{Hg}^{2+}$ , and millimolar NaF, and was stable at neutral pH in detergent and high glycerol concentration. The results of this and previous work (Belfrage, P., Fredrikson, G., Nilsson, N. Ö., and Strålfors, P. (1980) *FEBS Lett.* 111, 120-124; Nilsson, N. Ö., Strålfors, P., Fredrikson, G., and Belfrage, P. (1980) *FEBS Lett.* 111, 125-130) demonstrate modulation of hormone-sensitive lipase activity by phosphorylation *in vitro* as well as in the intact fat cell and indicate that this is a significant mechanism for the hormonal short term regulation of adipose tissue lipolysis.

Fatty acid mobilization from adipose tissue is regulated by hormone-sensitive lipase, the enzyme catalyzing the rate-limiting step in the hydrolysis of stored triacylglycerol (2). The hormonal regulation of the enzyme (3-5) was early proposed to be mediated by cAMP-dependent phosphorylation in analogy with the hormonal regulation of glycogen degradation (6, 7). Supporting this, particulate preparations of hormone-sensitive lipase were activated by cAMP-dependent protein kinase (8-10) and deactivated by protein phosphatases (11). However, purified lipase has not been available to experimentally verify this proposed mechanism.

We recently prepared hormone-sensitive lipase in detergent-solubilized, partially purified form, tentatively identified it with a polypeptide of  $M_r = 86,000$ <sup>1</sup> by SDS-PAGE<sup>2</sup>, and demonstrated its cAMP-dependent, protein kinase-catalyzed phosphorylation and activation (12). Extending these findings, we describe in this report a procedure for detergent solubilization and extensive purification of hormone-sensitive lipase from rat adipose tissue resulting in a stable enzyme of 50% protein purity. The previous identification of the enzyme protein is confirmed and enhancement of enzyme activity by phosphorylation with cAMP-dependent protein kinase is established. Substrate specificity, inhibition characteristics, and some additional properties of the purified enzyme are described and compared with those of other tissue lipases.

### EXPERIMENTAL PROCEDURES

**Materials**—[ $^3\text{H}$ ]oleoyl-labeled acyl- and acylalkylglycerols, monooleoyl [ $^3\text{H}$ ]glycerol, and cholesterol [ $^3\text{H}$ ]oleate were synthesized and purified as previously described (13-16). Soybean phosphatidylcholine (Epicuron 200, >99% pure) was from L. Meyer (Hamburg, West Germany). Phosphatidylinositol (Epicuron 510) was purified by ion exchange chromatography on CM-cellulose. [ $\gamma\text{-}^{32}\text{P}$ ]ATP was synthesized and purified as in Ref. 17. Bovine serum albumin (crystalline, defatted) was from Sigma; protease inhibitors, leupeptin, and pepstatin were from Peptide Institute, Inc. (Osaka, Japan); QAE-Sephadex A-25 and Sephacryl S-200 were from Pharmacia; hydroxyapatite (spheroidal) was from BDH. The catalytic subunit of cAMP-dependent protein kinase was purified to near homogeneity from rat adipose tissue.<sup>3</sup> Monoacylglycerol lipase (18), >95% protein purity, was prepared from rat adipose tissue by Dr. Hans Tornqvist. The specific inhibitor protein of cAMP-dependent protein kinase (19) was a generous gift from Dr. Philip Cohen, University of Dundee, Great Britain; [ $^{25}\text{I}$ ]-labeled lipoprotein lipase (bovine milk) was from Dr. Gunilla Bengtsson, University of Umeå, and rabbit  $\beta_2$ -microglobulin was from Dr. Bo Åkerström, University of Lund, Sweden.

Male Sprague-Dawley rats, 200-220 g, were from Anticimex (Swe-

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<sup>1</sup> The  $M_r$  of the enzyme was found to be 84,000 rather than 86,000 (12) with the reference proteins and the sodium dodecyl sulfate-polyacrylamide gel electrophoresis system used in this work.

<sup>2</sup> The abbreviations used are: SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; DFP, diisopropyl fluorophosphate; pH 5.2 ppt, pH 5.2 precipitate fraction; QAE-, diethyl-(2-hydroxypropyl)aminoethyl-.

<sup>3</sup> P. Strålfors, G. Fredrikson, N. Ö. Nilsson, and P. Belfrage, manuscript in preparation.



den) and were fed a standard commercial pellet diet until killed. Fat pads were immediately removed and either processed directly or frozen in homogenization solution ("Results") in liquid N<sub>2</sub> and stored at -70 °C until further use.

**Detergents**—Alkyl polyoxyethylene ethers with the general formula C<sub>12</sub>H<sub>25</sub>(OCH<sub>2</sub>CH<sub>2</sub>)<sub>n</sub>OH, abbreviated to C<sub>n</sub>E<sub>x</sub>, were used. C<sub>12</sub>E<sub>12</sub>, a heterogeneous emulsifier, Berol 058, was from Berol Kemi AB (Stenungsund, Sweden); homogeneous C<sub>12</sub>E<sub>6</sub> was from Nikko Chemicals (Tokyo, Japan), while C<sub>12</sub>E<sub>6</sub>, a mixture of C<sub>12</sub>E<sub>6</sub> (<25%), C<sub>12</sub>E<sub>8</sub> (>50%), and C<sub>12</sub>E<sub>10</sub> (<25%), was synthesized as in Ref. 20. C<sub>12</sub>E<sub>12</sub> and C<sub>12</sub>E<sub>6</sub> have a critical micellar concentration at 25 °C of approximately 0.1 mM and C<sub>12</sub>E<sub>8</sub> has a critical micellar concentration of 10 mM (21). Heterogeneous [<sup>14</sup>C]alkyl-labeled C<sub>12</sub>E<sub>12</sub> (or [<sup>14</sup>C]detergent) was synthesized and purified as will be described elsewhere.<sup>6</sup> All detergents were deionized in concentrated aqueous solution on a mixed ion exchange resin and stored at -20 °C.

**Affinity Chromatography Gel**—Ultrogel AcA 34 (manufactured by l'Industrie Biologique Française, Gennevilliers, France) was obtained from LKB. Gel produced before 1977 contains soybean oil triacylglycerol, 0.3 mg/g, wet weight, remaining from the manufacturing procedure. Triacylglycerol (approximately 0.2 mg/g) can be included into more recent gel batches as follows. After washing the gel with water and 75% ethanol and sucking it dry, soybean oil triacylglycerol (30 g/100 ml of gel) in ethanol is added and the mixture is stirred overnight, all procedures at room temperature. It is poured quickly into 10 volumes of 1 M NaCl and extensively washed with 1 M NaCl and excess lipid is removed. The gel is then washed with several volumes of the equilibration buffer with C<sub>12</sub>E<sub>12</sub> described in the legend for Fig. 3.

**Enzyme Assays**—Hormone-sensitive lipase activity was determined with tri[<sup>3</sup>H]oleoylglycerol, di[<sup>3</sup>H]oleoylglycerol, 1(3)-mono[<sup>3</sup>H]oleoyl-2-O-oleoylglycerol, and monooleoyl[<sup>3</sup>H]glycerol substrates, emulsified with phospholipids (phosphatidylinositol:phosphatidylcholine, 1:3, w/w) by sonication, essentially as previously described (14, 15, 22). Cholesterol [<sup>3</sup>H]oleate, 0.45 mM, was emulsified with an equimolar amount of the same phospholipid mixture. Monoacylglycerol lipase (18) was determined with micellar monooleoyl[<sup>3</sup>H]glycerol (13) and lipoprotein lipase was determined with emulsified tri[<sup>3</sup>H]oleoylglycerol (enzyme was prepared as acetone ether extracts) (23). Lipoprotein lipase contributed less than 2 and 10% to the activity measured in the assays for hormone-sensitive lipase with di- and trioleoylglycerol, respectively (equal amounts of triacylglycerol lipase activity of both enzymes were used). Unless otherwise stated, incubation time was always 30 min at 37 °C. Assay of hormone-sensitive lipase required dilution, in the presence of 0.2 mg/ml of albumin, of samples to noninhibitory detergent concentration. Hormone-sensitive lipase activity in crude adipose tissue preparations was also determined by pH-stat titration. Conditions were as for determination of fatty acid release from intact adipocytes (24) but with 25 μM dithioerythritol and 20 μg/ml of leupeptin in the incubation medium. Substrates used were either endogenous tissue triacylglycerol or added emulsified 1(3)-monooleoyl-2-O-oleoylglycerol at concentrations which were not rate-limiting. One unit of enzyme activity is equivalent to 1 μmol of fatty acid released/min at 37 °C, and specific activity is expressed as units/mg of protein.

Cyclic AMP-dependent protein kinase was assayed with protamine (25) as substrate and protein phosphatase activity was assayed with [<sup>32</sup>P]phosphoprotamine (26, 27) as substrate.

**Phosphorylation and Activation of Hormone-sensitive Lipase**—Hormone-sensitive lipase was phosphorylated by incubation with the purified catalytic subunit of cAMP-dependent protein kinase from rat adipose tissue (2 μg/ml) in 0.1 M Tris-HCl, pH 7.0,<sup>6</sup> containing 10 mM MgCl<sub>2</sub>, 1 mM dithioerythritol, 1 mg/ml of albumin, 0.2 mM [γ-<sup>32</sup>P]ATP (10<sup>4</sup> cpm/pmol) for 30 min at 37 °C. Phosphorylation was terminated by protein precipitation in 10% trichloroacetic acid. <sup>32</sup>P incorporation was determined after SDS-PAGE and autoradiography, quantitated by scanning densitometry (see below) or by liquid scintillation counting of the excised protein band dissolved in H<sub>2</sub>O.

Activation of enzyme was performed as above, except that unlabeled ATP was used and incubations were terminated by addition of 4 volumes of ice-cold 5 mM EDTA, 1 mM dithioerythritol, pH 7.0. Enzyme activity was assayed toward 0.5 mM trioleoylglycerol (see above) in 20 mM Tris-HCl, pH 8.0, 5 mM NaCl for 30 min at 37 °C. In

controls, ATP was omitted.

**[<sup>3</sup>H]DFFP Labeling of Hormone-sensitive Lipase**—Enzyme was incubated with [<sup>3</sup>H]DFFP (14·10<sup>4</sup> dpm/pmol) (Radiochemical Centre, Amersham, Great Britain) at indicated concentrations for 30 min at 37 °C. After addition of carrier protein, 10 μg of β<sub>2</sub>-microglobulin, incubations were terminated by 1 volume of 20% (w/v) trichloroacetic acid and prepared for SDS-PAGE (see below). [<sup>3</sup>H]DFFP incorporation was determined after SDS-PAGE, fluorography (see below), and scanning densitometry or by liquid scintillation counting after elution of the protein from excised gel slices in Liposolve and Lipoluma (Lumac, Basel, Switzerland).

**Concentration of Enzyme**—Enzyme was concentrated 10-fold by pressure ultrafiltration at 4 °C using an Amicon Diaflo PM 10 filter (NaCl was kept at least at 50 mM to prevent enzyme aggregation). Detergent in the enzyme preparation was concentrated to the same extent.

By reversible adsorption on hydroxyapatite, enzyme was concentrated without concomitant concentration of detergent, and detergents could also be exchanged. The enzyme preparation was dialyzed overnight at 4 °C against 50% (w/v) glycerol, 1 mM dithioerythritol, 10 μg/ml of leupeptin. It was then applied to a hydroxyapatite column (0.1 g/ml of enzyme solution) equilibrated in 10 mM potassium phosphate, pH 7.0, 30% (w/v) glycerol, 1 mM dithioerythritol, 0.2% (w/v) C<sub>12</sub>E<sub>12</sub> or the same buffer but with 50% (w/v) glycerol, 2 mM C<sub>12</sub>E<sub>6</sub>. After sample application, the column was washed with 4 volumes of the equilibration buffer at a flow rate of 20–30 cm/h. The enzyme was eluted in the equilibration buffer with 0.5 or 0.3 M potassium phosphate, respectively.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**—SDS-PAGE was performed as described by Laemmli (28) with modifications (29). Enzyme samples were precipitated with trichloroacetic acid and extracted with solvent before electrophoresis (29). As reference proteins, phosphorylase *a* from rabbit muscle (*M*<sub>r</sub> = 94,000), human transferrin (*M*<sub>r</sub> = 76,700), bovine serum albumin (*M*<sub>r</sub> = 67,000), and ovalbumin (*M*<sub>r</sub> = 43,000) were used (all from Sigma). Protein bands were stained with Coomassie blue (Kenacid blue; BDH) and roughly quantitated by scanning densitometry at 633 nm (softer laser densitometer, model SL 504; Biomedical Instruments, Chicago).

**Autoradiography and Fluorography**—Stained and dried polyacrylamide gels were autoradiographed using Kodak X-Omat R film and an intensifying screen (DuPont) (30). For fluorography, stained gels were treated with sodium salicylate (31), dried, and autoradiographed as above.

**Protein Determination**—Protein was either monitored by absorbance at 280 nm or determined with a scaled down version (32) of the method of Lowry *et al.* (33) after trichloroacetic acid precipitation in detergent followed by extraction with diethyl ether:ethanol, 3:1 (v/v). Albumin was used as standard.

**Other Methods**—Phospholipids were determined as lipid phosphorus (34). Concentration of sodium chloride was measured by its conductivity. Mono[<sup>3</sup>H]oleoylglycerol in lipid extracts was isolated by thin layer chromatography on silicic acid in hexane:diethyl ether:methanol:acetic acid (90:20:3:2, v/v) and quantitated by liquid scintillation counting.

## RESULTS

Hormone-sensitive lipase activity was routinely assayed with monooleoyl-2-O-oleoylglycerol, a monoether analogue of dioleoylglycerol (unless otherwise stated, enzyme activity refers to this monoacylalkylglycerol hydrolase activity), since the enzyme hydrolyzes this substrate 10-fold faster than triacylglycerols. Moreover, monoacylalkylglycerol cannot be hydrolyzed to monoacylglycerols and thus cannot produce substrate for monoacylglycerol lipase. In preparations lacking this enzyme, the activity toward monoacylalkylglycerol was equal to the diacylglycerol hydrolase activity of the hormone-sensitive lipase (see below).

A representative purification is summarized in Table I.

**Step I, II, and III: Initial Fractionation of Crude Tissue Extracts**—Epididymal adipose tissue (approximately 1.1 g of tissue/rat) was homogenized in 2 volumes of 0.25 M sucrose, 1 mM EDTA, 1 mM dithioerythritol, 20 μg/ml of leupeptin, 1 μg/ml of pepstatin at pH 7.0 and 10 °C. The use of protease inhibitors was necessary when frozen fat was used to prevent rapid proteolytic degradation of the enzyme. Floating fat and

<sup>6</sup> G. Fredrikson, L. Krabich, and P. Belfrage, manuscript in preparation.

<sup>7</sup> Unless otherwise stated, pH values refer to the temperature at which solutions or buffers have been used.



TABLE I

Purification of hormone-sensitive lipase from rat adipose tissue

Enzyme was purified from 220 g of fat pads obtained from 200 rats. Enzyme activity was measured toward mono[<sup>3</sup>H]oleoylalkylglycerol substrate ("Experimental Procedures"). One unit of enzyme activity is equivalent to 1  $\mu$ mol of fatty acid produced/min at 37 °C.

| Purification step                             | Total activity<br>units | Specific activity<br>units/mg protein | Recovery<br>%    |
|-----------------------------------------------|-------------------------|---------------------------------------|------------------|
| I. Tissue homogenate                          | 160 <sup>a</sup>        | 0.049 <sup>a</sup>                    | 100              |
| II. 110,000 $\times$ g supernatant            | 100                     | 0.059                                 | 62               |
| III. pH 5.2 precipitate                       | 93                      | 0.22                                  | 58               |
| IV. Solubilized pH 5.2 precipitate            | 88                      | 0.21                                  | 55               |
| V. First gradient sievortive chromatography   | 44                      | 3.1                                   | 28               |
| VI. Second gradient sievortive chromatography | 23                      | 7.4                                   | 14               |
| VII. Concentration on hydroxyapatite          | 14                      | 7.2                                   | 8.8              |
| VIII. Affinity chromatography                 | 7.2 <sup>b</sup>        | 100 <sup>b,c</sup>                    | 4.6 <sup>b</sup> |

<sup>a</sup> Measured by pH-stat titration using unlabeled monooleoylalkylglycerol ("Experimental Procedures").

<sup>b</sup> Represents the combined enzyme from two identical chromatographies.

<sup>c</sup> Protein was measured after concentration of the enzyme on hydroxyapatite.

sedimented particulate material were removed after centrifugation at 110,000  $\times$  g for 45 min. After precipitation at pH 5.2 (4 °C, 30 min), the precipitate formed was collected by centrifugation and suspended in (0.25 ml/rat) 20 mM Tris-HCl, pH 7.0, 1 mM EDTA, 1 mM dithioerythritol, 10  $\mu$ g/ml of leupeptin. This pH 5.2 precipitate fraction could be stored at -70 °C for several months without loss of enzyme activity.

pH-stat titration, using monoacylalkylglycerol as substrate, showed that most of the enzyme activity of the homogenate was recovered in the 110,000  $\times$  g supernatant; some remained with the floating fat (25%) and less than 10% sedimented in the high speed centrifugation.

**Step IV: Detergent Solubilization**—The enzyme in the pH 5.2 ppt and the other crude tissue extracts resided in protein-lipid aggregates of high molecular weight as revealed by gel chromatography. The lipids (5 mg/ml of pH 5.2 ppt) of these aggregates were mainly phospholipid with some cholesterol, triacylglycerols, and unesterified fatty acid.

The enzyme was solubilized with detergent as follows: 13% (w/v) sucrose, 65 mM NaCl, 1 mM EDTA, 1 mM dithioerythritol (final concentrations) were dissolved in 50 ml of pH 5.2 ppt. This solution was added in portions, with light sonication, to concentrated detergent solution: 69 ml of 12% (w/v) C<sub>12</sub>E<sub>12</sub> and <sup>14</sup>C detergent (1.10<sup>6</sup> cpm) in 30 mM Tris-HCl, pH 7.97.

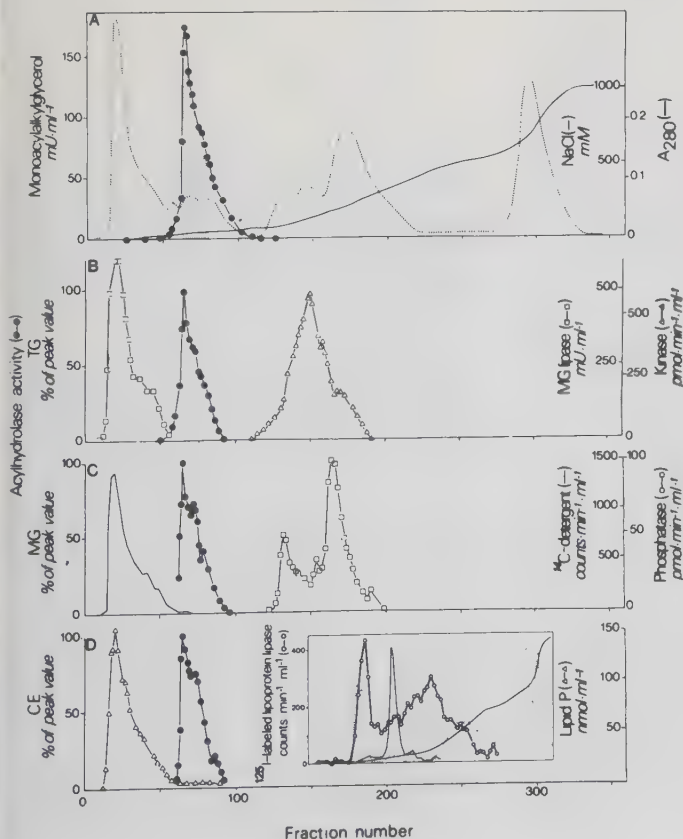


FIG. 1. First gradient sievortive chromatography. QAE-Sephadex (10  $\times$  40 cm) was equilibrated successively at 10 °C with 10 volumes of 0.1 M Tris-HCl, 10 volumes of 40 mM Tris-HCl, 15 mM NaCl and 2 volumes of 40 mM Tris-HCl, 20% (w/v) glycerol, 1 mM dithioerythritol, 0.2 mM EDTA, 0.2% (w/v) C<sub>12</sub>E<sub>12</sub>, 15 mM NaCl, all pH 7.97. The sample, 130 ml of solubilized pH 5.2 ppt, was applied (flow rate, 300 ml/h) followed by 200 ml of final equilibration buffer but with 65 mM NaCl, 5% (w/v) sucrose and eluted with the same buffer without sucrose. A 65–1000 mM NaCl gradient was then applied. Glycerol with 25 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM dithioerythritol, 10  $\mu$ g/ml of leupeptin (5.7 ml) was added continuously to each column fraction (14.4 ml). TG, MG, CE, emulsified tri-, monoacylglycerol, and cholesterol ester used as substrates (detergent inhibition prevented the measurement of absolute values); MG lipase, monoacylglycerol lipase; kinase, cAMP-dependent protein kinase; phosphatase, protein phosphatase; lipid P, lipid phosphorus. Inset, same chromatography (scaled down to a column of 2.6  $\times$  90 cm) of solubilized pH 5.2 ppt containing <sup>125</sup>I-lipoprotein lipase. Conditions as above. Except for <sup>125</sup>I-lipoprotein lipase, illustrated parameters can be identified from comparison with A.

The mixture was then sonicated at 10 °C until almost clear visually. Insoluble material was removed by centrifugation at  $15,000 \times g$  for 20 min. The final detergent concentration (6.5%, w/v) was chosen to correspond to at least 5 detergent micelles/protein molecule (*cf.* Ref. 21). Detergent was included in all solutions in subsequent purification steps.

**Step V: First Gradient Sievortive Chromatography**—Conventional ion exchange chromatography resulted in large losses of enzyme, probably due to irreversible aggregation of proteins concentrated by adsorption to the matrix. This problem was overcome by fractionation with gradient sievortive ion exchange chromatography (detailed description of this technique in Ref. 35), which allowed the proteins to interact with the matrix without being bound and concentrated.

The solubilized pH 5.2 ppt was chromatographed according to this technique on a short QAE-Sephadex column (Fig. 1). The column was developed by the inherent 15–65 mM NaCl gradient, formed by applying sample and eluting in 65 mM NaCl (*cf.* Ref. 35). Hormone-sensitive lipase was eluted at 40 mM NaCl (Fig. 1A). The elution patterns for all acylhydrolase activities were the same except for the major part of the activity toward monoacylglycerols, due to monoacylglycerol lipase that eluted with the unretained protein peak (Fig. 1B).

No other acylhydrolase activity was detected when the entire column eluate was tested with all four acylhydrolase substrates at neutral and acid pH. Total enzyme recovery was more than 70% at this step. The enzyme was stable only at neutral pH. By lowering the pH of the chromatography toward neutrality, enzyme recovery could be increased to almost 100% at the expense of decreased resolution. Additional inactivation was caused by the pH increase due to salt gradient effects on the ion exchange gel matrix (Fig. 2A; not illustrated in Fig. 1). The pH inactivation was minimized by continuously bringing column effluent to pH 7 in 50% (w/v) glycerol.

At this step, the enzyme was separated from the bulk of sample detergent ( $^3\text{H}$  detergent; Fig. 1C) and phospholipids (Fig. 1D) (neutral lipids also eluted with unretained proteins). Lipoprotein lipase activity, known to be present in the pH 5.2 ppt, could not be measured in the column eluate, probably due to detergent inhibition.  $^{125}\text{I}$ -Lipoprotein lipase added to the pH 5.2 ppt, solubilized and subjected to gradient sievortive chromatography, was partly eluted with the unretained proteins (Fig. 1D, inset). Most of the enzyme, however, was eluted over a whole column volume, presumably aggregated (total yield was 87%). The small amount (<10% of initial) eluting with hormone-sensitive lipase at this step was negligible (<0.5% of initial) after the second gradient sievortive chromatography.

Cyclic AMP-dependent protein kinase and protein phosphatase activities were eluted well separated from hormone-sensitive lipase (Fig. 1, B and C). Very low levels of the catalytic subunit of cAMP-dependent protein kinase and a cAMP-independent protein kinase activity contaminated hormone-sensitive lipase fractions but were completely removed after the second gradient sievortive chromatography (not illustrated).

**Step VI: Second Gradient Sievortive Chromatography**—Indicated fractions from the first gradient sievortive chromatography (Fig. 1A, shaded area) were concentrated approximately 10-fold by pressure ultrafiltration ("Experimental Procedures") and dialyzed at 10 °C against the elution buffer with 50 mM NaCl used at the second gradient sievortive chromatography (legend for Fig. 2). The enzyme was then immediately (due to the pH inactivation) applied to a long QAE-Sephadex column and eluted with a preformed salt gradient of gentle slope (Fig. 2).

Hormone-sensitive lipase eluted at 40 mM NaCl in 70% total

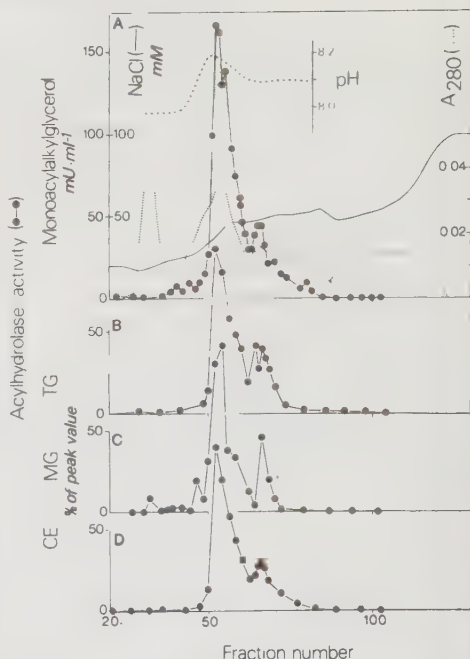


Fig. 2. Second gradient sievortive chromatography. QAE-Sephadex ( $5 \times 98$  cm) was equilibrated as described for Fig. 1 but with 20 mM instead of 15 mM NaCl. Succeeding a salt gradient, 130 ml of 20–50 mM NaCl in final equilibration buffer, the sample (63 ml, concentrated and dialyzed fractions from the first gradient sievortive chromatography) was applied (flow rate, 100 ml/h) followed by 50 mM NaCl in the same buffer. Adsorbed proteins were then eluted by increasing NaCl to 100 mM. Fraction volume was 14.5 ml (before addition of 4.8 ml of acid glycerol; see Fig. 1). Abbreviations are as in Fig. 1. A, pH measured at 10 °C in the eluate of a column run under identical conditions.

yield (Fig. 2A). The elution patterns for all acylhydrolase activities were similar (Fig. 2). The enzymological properties of the second enzyme peak, including its phosphorylatability, could not be distinguished from those of the main enzyme peak. Thus, it probably reflected enzyme charge heterogeneity.

The protein purity of the enzyme after this step was 3–4%. At this stage of purification, the enzyme preparation completely lacked monoacylglycerol lipase, lipoprotein lipase, and the activities potentially involved in the regulation of hormone-sensitive lipase, those of protein kinase and protein phosphatase.

**Step VII: Affinity Chromatography**—Indicated fractions from the second gradient sievortive chromatography were dialyzed and concentrated on hydroxyapatite ("Experimental Procedures") in a total yield of 75%. The most concentrated enzyme (80% of total) was then subjected to affinity chromatography on a triacylglycerol-containing polyacrylamide-agarose gel (Ultragel AcA 34; "Experimental Procedures") in  $\text{C}_{12}\text{E}_{10}$  detergent (Fig. 3).

The enzyme was eluted (70% total yield) immediately before the column total volume and the high phosphate applied with the sample (Fig. 3A). The elution patterns for all four acylhydrolase activities were again the same (Fig. 3), indicating

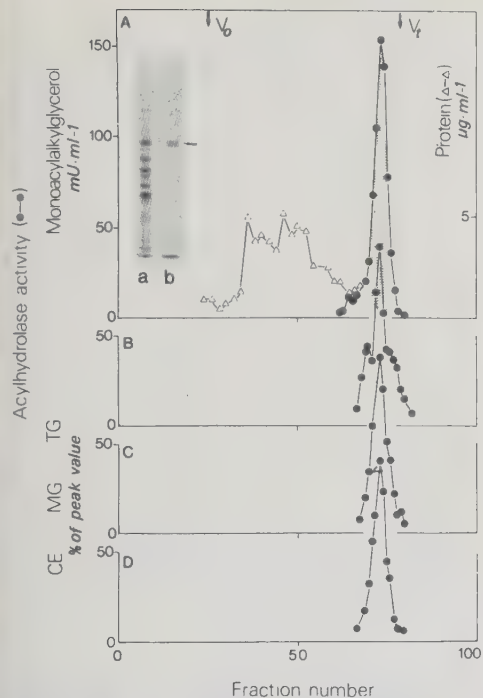


Fig. 3. Affinity chromatography. Ultrogel AcA 34 ( $2.6 \times 89$  cm) containing triacylglycerol was equilibrated in 1 volume of 5 mM potassium phosphate, pH 7.0, 30% (w/v) glycerol, 1 mM dithioerythritol, 0.15 M NaCl, 0.2% (w/v)  $C_{13}E_{12}$ , followed by 2 volumes of the same buffer with 0.8% (w/v)  $C_{12}E_6$  instead of  $C_{13}E_{12}$ . Column dimensions and other conditions were chosen for optimal resolution; therefore, the concentrated enzyme from the second gradient sievortype chromatography was fractionated one-half at a time. The sample, 8.8 ml, was followed by 12 ml of sample buffer (0.5 M potassium phosphate, pH 7.0, 30% (w/v) glycerol, 1 mM dithioerythritol, 0.2% (w/v)  $C_{13}E_{12}$ ) and the enzyme eluted with the equilibration buffer with  $C_{12}E_6$ . Flow rate was 23 ml/h, fraction volume, 6.1 ml and temperature,  $4^\circ\text{C}$ .  $C_{13}E_{12}$  was added to each column fraction to 0.1% (w/v) to stabilize the enzyme. Abbreviations are as in Fig. 1. *Inserts*, Coomassie blue-stained SDS-PAGE slab gels of (a) pooled enzyme from Step VII (approximately 30  $\mu\text{g}$ ) and (b) pooled enzyme from Step VIII concentrated on hydroxyapatite (approximately 4  $\mu\text{g}$ );  $\beta_2$ -microglobulin (10  $\mu\text{g}$ ) was added before precipitation of sample.

that they were all due to hormone-sensitive lipase. The pattern of eluted protein could not be monitored by its absorbance at 280 nm due to its low concentration and interfering trace impurities in the  $C_{12}E_6$  detergent. Protein values given in Fig. 3A (measured according to Lowry *et al.* (33) as modified by Tornqvist and Belfrage (32)) should only be considered as approximate since the high concentration of  $C_{12}E_6$  detergent partly prevented precipitation of the protein. This precluded a direct quantitation of the minute amounts of protein in the enzyme fractions. However, the mean protein concentration of the pooled enzyme (Fig. 3A, shaded area) was calculated to approximately 1  $\mu\text{g}/\text{ml}$  by protein determination after concentration and  $C_{12}E_6$  removal on hydroxyapatite (see below).

In the pooled enzyme, the predominant protein species, the  $M_r = 84,000$  polypeptide identified below with the lipase, was

enriched from approximately 4 to 45% of stainable protein by the affinity chromatography step (Fig. 3A, *Inserts a* and *b*). SDS-PAGE performed on consecutive fractions over the enzyme peak showed that this polypeptide selectively coeluted with enzyme activity (*cf.* Ref. 12), thereby separating from the main protein contaminant with slightly higher electrophoretic mobility (data not shown).

The mechanism of adsorption of the enzyme on the Ultrogel AcA 34 is not clear. The presence of triacylglycerol in the gel matrix was, however, necessary, as was equilibration in  $C_{12}E_6$  detergent instead of the usual  $C_{13}E_{12}$ , possibly reflecting the difference in critical micellar concentration between these two detergents. True affinity interaction between enzyme and immobilized substrate is certainly an important part of the overall process, but nonspecific hydrophobic interactions and polar interactions probably also determine the behavior of the enzyme and contaminating proteins eluting with the enzyme (*cf.* Ref. 36).

Overall recovery of enzyme activity after this final purification step was 4–5% (range of six different purifications) but, as discussed below, due to pH inactivation, enzyme protein yield was higher. The specific activity of the final enzyme preparation was 100 units/mg of protein (Table I), varying between 83–113 in three different purifications.

Enzyme activity was labile in  $C_{12}E_6$ , in which it was eluted from the affinity chromatography column. A stable working preparation, referred to as purified enzyme in the following, was obtained by concentration with detergent exchange of indicated fractions (Fig. 3A) on hydroxyapatite ("Experimental Procedures"). After this procedure, *i.e.* in 0.3 M potassium phosphate, pH 7.0, 50% (w/v) glycerol, 1 mM dithioerythritol, 2 mM  $C_{12}E_6$ , enzyme activity was stable (>90%) for at least 2 months at  $-70^\circ\text{C}$ . Storage in small aliquots prevented loss of activity otherwise resulting from repeated thawing and freezing. The enzyme was also stable for several hours at  $37^\circ\text{C}$ , but much less so after 100-fold dilution in the absence of glycerol and detergent.

**Purity of Enzyme**—The inhibition of hormone-sensitive lipase by DFP (see below) implies a covalent modification of a catalytically essential serine residue (37). The selective enrichment, during the purification, of [ $^3\text{H}$ ]DFP label in the  $M_r = 84,000$  polypeptide (Fig. 4A) and its exclusive labeling in the purified enzyme preparation (Fig. 4A) confirmed its identification with the hormone-sensitive lipase (12). In addition, lipase-substrate binding was directly related to the  $M_r = 84,000$  polypeptide because this binding selectively and reversibly protected it from modification by DFP. In the presence of emulsified acylglycerol, enzyme from Step VII was incubated with nonlabeled DFP. After successive removal of the DFP and substrate by microcentrifuge desalting (38) and flotation, respectively, [ $^3\text{H}$ ]DFP incorporation into the other polypeptides decreased to less than 10%, whereas in the  $M_r = 84,000$  polypeptide, the incorporation was unchanged when compared to controls pretreated without nonlabeled DFP.

Furthermore, the functional relationship between the enzyme and the  $M_r = 84,000$  polypeptide was demonstrated by its selective phosphorylation with enhancement of enzyme activity (see below). In the second gradient sievortype chromatography and subsequent steps, only this polypeptide was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase from rat adipose tissue and [ $\gamma\text{-}^{32}\text{P}$ ]ATP-Mg (Fig. 4B).

The purity of the enzyme protein, the  $M_r = 84,000$  polypeptide, estimated by scanning densitometry of Coomassie blue-stained proteins after SDS-PAGE, was 45% (Fig. 4C), varying between 40–60% with six different batches of purified enzyme.

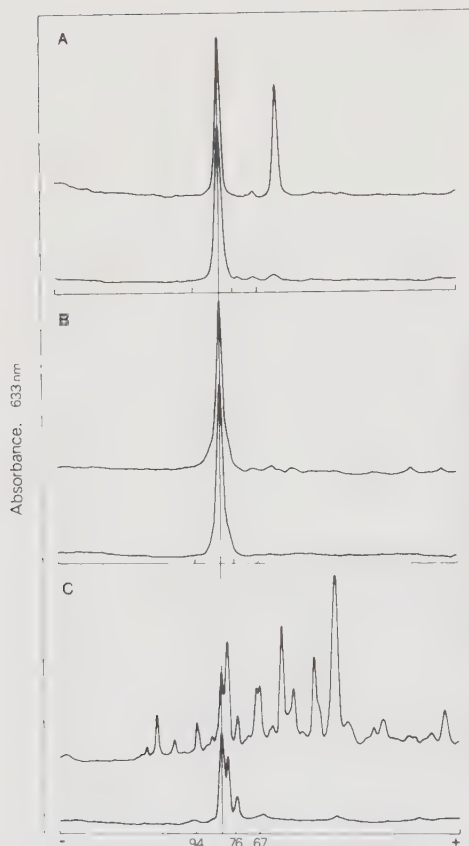


FIG. 4. Scanning densitograms from SDS-PAGE of hormone-sensitive lipase. Hormone-sensitive lipase from Step VII, 60  $\mu$ g of protein (upper), and the purified enzyme, 4  $\mu$ g of protein (lower), were analyzed by SDS-PAGE and scanning densitometry after (A) [ $^3$ H]DFP labeling and fluorography ("Experimental Procedures"), (B) phosphorylation and autoradiography ("Experimental Procedures"), and (C) Coomassie blue staining of protein. Migrating positions of reference proteins and hormone-sensitive lipase are indicated ( $k = 10^3$ ).

**Relative Acylhydrolase Activities**—The four acylhydrolase activities eluted almost identically in the chromatographies (Figs. 1–3), indicating that they were all due to hormone-sensitive lipase. However, only the activity toward monoacylalkylglycerol (and diacylglycerol) could be measured under conditions of minimal detergent inhibition. Enzyme from different stages of purification was therefore concentrated on hydroxyapatite, to allow dilution to noninhibitory detergent concentration (approximately 0.0002% (w/v)  $C_{12}E_8$  or 3  $\mu$ M  $C_{12}E_8$ ).

The enzyme activity "profile," approximately 1:10:4:1.5 for tri-, di-, and monoacylglycerol and cholesterol ester substrate, was quite similar over the successive chromatographies, monoacylalkylglycerol and diacylglycerol lipase activities being equal (Fig. 5B). At the stages of purification prior to the chromatographies, including the tissue homogenate (meas-

ured with pH-stat titration), activities toward tri-, di-, and especially monoacylglycerol were relatively higher (Fig. 5A). The higher activity toward the last substrate was due to monoacylglycerol lipase (cf. Fig. 1, B and C). However, this enzyme was also responsible for increased fatty acid release from tri- and diacylglycerol with crude tissue preparations (Fig. 5A) by hydrolysis of labeled monoacylglycerols formed as reaction products after hormone-sensitive lipase action on the first ester bonds. This was indicated by the discrepancy between the diacylglycerol and monoacylalkylglycerol hydrolyase activities in crude tissue fractions (Fig. 5A) and by the fact that labeled monoacylglycerol was found, by thin layer chromatography, to be a large part of the reaction products during hydrolysis of tri- and diacylglycerol with purified enzyme (up to 50% with the latter substrate) but much less with enzyme preparations containing monoacylglycerol lipase (only unesterified  $^3$ H-fatty acids are isolated and quantitated by the assay procedure used). Addition of pure monoacylglycerol lipase to enzyme preparations at different stages of purification, as expected, had little additional effect on hydrolysis of tri- and diacylglycerol by crude enzyme (Fig. 5, Step III enzyme) but increased these activities with purified enzyme (Fig. 5, Steps V and VIII enzymes) to almost the same level as with crude enzyme. Labeled monoacylglycerol no longer accumulated. Thus, the stated enzyme activity profile is likely to be valid for hormone-sensitive lipase *per se*.

**Effect of Inhibitors**—Enzyme activity toward the illustrated substrates (Table II) was 50% inhibited by DFP, NaF, and  $Hg^{2+}$  at approximately the same inhibitor concentrations, both

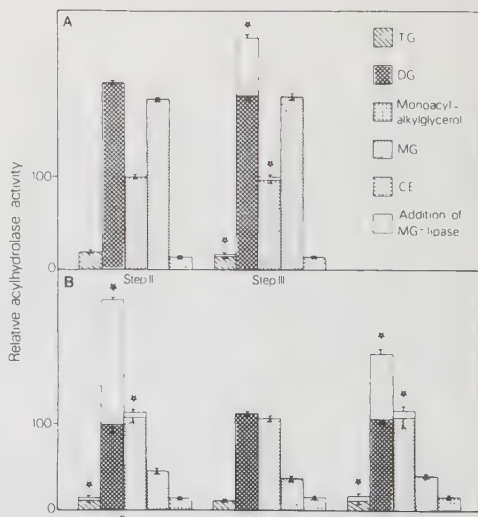


FIG. 5. Relative acylhydrolase activities at different steps of purification. Hormone-sensitive lipase was measured toward the indicated substrates (30 min at 37  $^{\circ}$ C). Enzyme was from the indicated purification steps (see Table I). Steps V and VIII enzymes were first concentrated on hydroxyapatite ("Experimental Procedures"). When required, enzyme samples were diluted to 3  $\mu$ M detergent (final concentration). Monoacylglycerol lipase (approximately 4 ng/incubation) was added to incubations with tri- and dioleoylglycerol and monoacylalkylglycerol substrates (as indicated by \*) with Steps III, V, and VIII enzymes. Vertical bars indicate S.E. ( $n = 5$ ). Enzyme activity against monoacylalkylglycerol was set to 100 in each step, and other activities were expressed relative to this. Abbreviations are as in Fig. 1.



TABLE II

## Inhibitors of hormone-sensitive lipase activity

Enzyme, diluted 500-fold to approximately 1 ng/incubation (100  $\mu$ l), was preincubated with varying concentrations of inhibitors for 30 min at 37 °C. Substrate was then added and the incubation continued for another 30 min.

| Inhibitor and Substrate | Inhibitor concentration <sup>a</sup> at 50% inhibition |                 |
|-------------------------|--------------------------------------------------------|-----------------|
|                         | pH 5.2 ppt enzyme                                      | Purified enzyme |
| DFP                     |                                                        |                 |
| Trioleoylglycerol       | 12                                                     | 9               |
| Cholesterol oleate      | 11                                                     | 10              |
| Monooleoylalkylglycerol | 9                                                      | 8               |
| NaF                     |                                                        |                 |
| Trioleoylglycerol       | 25                                                     | 25              |
| Cholesterol oleate      | 25                                                     | 25              |
| Monooleoylalkylglycerol | 5                                                      | 4               |
| Monooleoylglycerol      | — <sup>b</sup>                                         | 3               |
| HgCl <sub>2</sub>       |                                                        |                 |
| Trioleoylglycerol       | 11                                                     | 11              |
| Cholesterol oleate      | 12                                                     | 12              |
| Monooleoylalkylglycerol | 12                                                     | 12              |

<sup>a</sup> Inhibitor concentration is micromolar except for values with NaF, which are millimolar.

<sup>b</sup> Could not be determined due to the presence of monoacylglycerol lipase.

with the purified lipase and with the pH 5.2 ppt preparation. The only exception was due to presence of monoacylglycerol lipase in the latter preparation, since this enzyme is not inhibited by NaF (18). The varying effect of the NaF inhibition with different substrates may indicate that it did not interact directly with the enzyme protein. Inhibition by DFP was dependent on the conditions of incubation with enzyme. Endogenous substrate (triacylglycerol) in crude tissue preparations, *e.g.* pH 5.2 ppt, protected the enzyme catalytic site from DFP modification; thus, millimolar DFP concentrations were required to significantly inhibit the enzyme when incubated in concentrated form (the pH 5.2 ppt data in Table II reflect some remaining protection, although the preparation was diluted 500-fold). Also with purified enzyme, dilution affected the sensitivity to DFP inhibition; with more concentrated enzyme, severalfold higher DFP concentrations were needed for the same effect (see below; Fig. 6).

While it was difficult to inhibit the enzyme in the crude tissue homogenate with DFP for the reasons described above, it was more than 90% inhibited by 200  $\mu$ M Hg<sup>2+</sup>, as measured by pH-stat titration of fatty acid release from endogenous lipids or added emulsified monoacylalkylglycerol or diacylglycerol. Preincubation of enzyme in 1.5 M NaCl at 37 °C had no significant effect on crude or purified enzyme. These inhibition characteristics, which agree with previous findings (7, 12, 39), indicate that hormone-sensitive lipase sustained by monoacylglycerol lipase accounted for most, possibly all, enzyme activity measured in the crude tissue preparations.

Detergents inhibited all acylhydrolase activities but to a variable degree, being more pronounced with tri- than with di- and monoacylated substrates. Inhibition also varied with the specific conditions in each assay (glycerol concentration, type of enzyme preparation). Therefore, determination of enzyme activity required careful attention to detergent concentration in the assay (see above).

**Specific Activity**—From Table I and Fig. 4C, the specific activity was calculated to be 220 units/mg of enzyme protein. However, this value was probably underestimated due to enzyme inactivation, mainly pH inactivation during the gradient sievortype chromatographies. Catalytically active enzyme has therefore been estimated. DFP inhibition and [<sup>3</sup>H]DFP labeling of purified enzyme varied similarly with

DFP concentration (Fig. 6), indicating that only catalytically active enzyme molecules were modified (*cf.* Ref. 37). From the enzyme activity inhibited by the incorporation of a particular amount of [<sup>3</sup>H]DFP (Fig. 6) and assuming that a single serine residue per enzyme molecule was modified (37), a molar specific activity of approximately 600 mol/s/mol of *M<sub>r</sub>* = 84,000 polypeptide at 37 °C could be calculated. This corresponds to a specific activity of 400–500 units/mg of enzyme protein. These data and the final yield of enzyme activity of 4.5% (Table I) thus suggest an overall yield of enzyme protein around 10%.

**Phosphorylation and Activation of Hormone-sensitive Lipase**—It was demonstrated above (Fig. 4B) that purified hormone-sensitive lipase was phosphorylated with the catalytic subunit of cAMP-dependent protein kinase and ATP-Mg. Under the same conditions, enzyme activity increased approximately 2-fold (Fig. 7), with a similar degree of activation at all stages of enzyme purification. In the pH 5.2 ppt, an endogenous cAMP-dependent protein kinase activated the

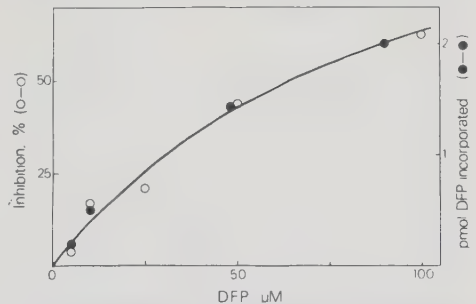


Fig. 6. Modification and inhibition of hormone-sensitive lipase by DFP. Concentrated purified enzyme (12 units/ml) was incubated with DFP and [<sup>3</sup>H]DFP (0.1 ml of enzyme) without further dilution ("Experimental Procedures"). After a 100-fold dilution, enzyme activity was measured with monoacylalkylglycerol substrate [<sup>3</sup>H]DFP incorporation was determined, after SDS-PAGE, from eluted gel slices ("Experimental Procedures").

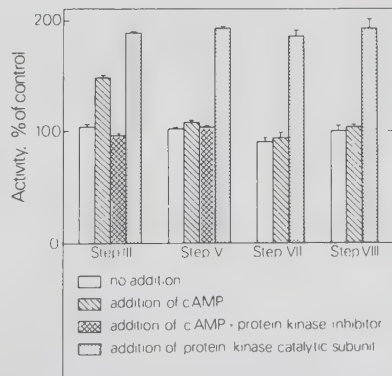


Fig. 7. Activation of hormone-sensitive lipase at different steps of purification. Hormone-sensitive lipase was activated with the indicated additions ("Experimental Procedures"). Enzyme activity is expressed as percentage of control. Vertical bars indicate S.E. (*n* = 5). Enzyme was from the indicated purification steps. Steps and VIII enzymes were first concentrated on hydroxyapatite ("Experimental Procedures").



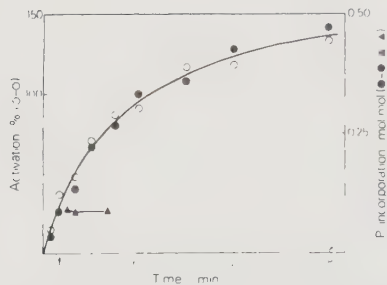


FIG. 8. Phosphorylation and activation of hormone-sensitive lipase. Purified enzyme was activated and phosphorylated as described under "Experimental Procedures." At indicated times, samples were taken for determination of enzyme activity and  $^{32}\text{P}$  incorporation. Inhibitor protein of cAMP-dependent protein kinase was added (arrow) and samples were withdrawn for determination of  $^{32}\text{P}$  incorporation (▲—▲). P incorporation is expressed as moles of P/mol of  $M_r = 84,000$  enzyme protein.

enzyme but addition of protein kinase catalytic subunit enhanced enzyme activity further. At all subsequent stages of purification, enzyme activation was completely dependent on exogenous protein kinase. Endogenous phosphorylation and activation were completely inhibited by the specific protein inhibitor of cAMP-dependent protein kinase.

To establish that activation was caused by phosphorylation, the time course of these parameters was followed with purified enzyme (Fig. 8). They closely correlated, with half-maximal effects on both after 35 min. The maximal activation of the enzyme approached 150% over control when approximately 0.5 mol of phosphate was incorporated/mol of enzyme protein ( $M_r = 84,000$ ). Addition of the inhibitor of cAMP-dependent protein kinase caused a prompt arrest of further phosphorylation of the purified enzyme (Fig. 8).

The ATP  $\gamma$ -phosphate incorporated was found to be stable in boiling acid and labile in boiling alkali (40), indicating phosphorylation of serine or threonine residues.

**Other Properties of the Enzyme.**—The purified enzyme had a broad pH optimum around pH 7. The activity against monoacylglycerol and cholesterol ester but not toward triacylglycerol was still high at pH 8, while below pH 6, the activity against all substrates declined quickly and disappeared below pH 5.

Its apparent  $M_r$  was 84,000 by SDS-PAGE under the conditions used (see "Experimental Procedures"). The apparent Stokes radius of the enzyme was 50 Å, as determined by gel chromatography on Sephacryl S-200 in  $\text{C}_{12}\text{E}_8$ , which might indicate an enzyme monomer in a detergent micelle or an enzyme dimer. The enzyme lost 50% of its activity by incubation for 30 min in 50% glycerol, 2 mM  $\text{C}_{12}\text{E}_8$ , at 70 °C but lost 50% of its activity at 30 °C when the glycerol was diluted 100-fold.

#### DISCUSSION

The enzyme, extensively purified in this work, was identified with hormone-sensitive lipase on the basis of several lines of evidence. It was purified from an adipose tissue preparation, the pH 5.2 ppt, the neutral lipase activity of which has been amply demonstrated to be due to this enzyme (10, 39, 41). The acylhydrolase activities copurified (Figs. 1–3) with a constant relation between the activities (Fig. 5), and no other lipases other than monoacylglycerol lipase and lipoprotein lipase were found to separate during the purification. Loss of enzyme can be accounted for by the pooling of enzyme fractions and

by inactivation, mainly pH inactivation. Moreover, the purified enzyme had the same inhibition characteristics as the pH 5.2 ppt lipase activity (Table II). The lipase could be activated by cAMP-dependent protein kinase (Fig. 7), a most important characteristic of hormone-sensitive lipase (8), and it had the inhibition properties of this enzyme (7). Most of the lipase activity of the crude homogenate was recovered in the pH 5.2 ppt (Table I), the only significant loss being that to the floating fat. However, this activity is likely also to be due to the same enzyme since  $^{32}\text{P}$ -labeled hormone-sensitive lipase (from *in vivo*  $^{32}\text{P}$ -labeled intact adipocytes (42)) distributed similarly (25–30% in the floating fat) and the inhibition properties were the same (90% inhibition by 200  $\mu\text{M}$   $\text{Hg}^{2+}$ , substrate protected from DFP modification). The original report characterizing hormone-sensitive lipase activity (7) did not exclude the possibility that this activity would be due to more than one lipase. The present work indicates that this may indeed be the case, since monoacylglycerol lipase contributed to the free fatty acid release measured in crude tissue preparations by hydrolyzing the monoacylglycerol formed.

The identification of the purified enzyme is further supported by quantitative considerations regarding its functional importance. Its activity in crude tissue homogenates can be compared with the lipolysis rates of intact tissue or fat cells. Glycerol release from whole fat pads (fed animals) equivalent to lipolysis rates of approximately 0.1  $\mu\text{mol}$  of fatty acids/min/g of fat pad before and 0.3 after maximal hormonal stimulation has been reported (43). Corresponding values for *in vivo* perfused parametrial adipose tissue (fasted animals) were 0.1 and 0.4, respectively (44). With intact fat cells from fed rats (220 g), we find a fatty acid release of less than 0.03  $\mu\text{mol}$ /min/ml of packed cells before and approximately 0.7 after maximal hormonal activation (*cf.* below). The rate of endogenous triacylglycerol hydrolysis in the crude homogenate used as the enzyme source in the purification described in this report (Table I) was 0.15  $\mu\text{mol}$  of fatty acid/min/g of adipose tissue. Thus, the lipase which has been purified may well account for the lipolysis rates obtained in intact tissue and fat cells.

The identification of the hormone-sensitive lipase with the protein of apparent minimum  $M_r$  of approximately 84,000 is established following several lines of evidence: (a) it was enriched through the successive stages of purification to almost 50% purity in the final preparation and selectively eluted together with enzyme activity in the affinity chromatography; (b) in the purified enzyme preparation, only this polypeptide was labeled with [ $^3\text{H}$ ]DFP, an inhibitor of the lipase activity, and this labeling and inhibition was shown to be modified by enzyme-substrate interaction; (c) only the  $M_r = 84,000$  polypeptide was  $^{32}\text{P}$  phosphorylated by incubation with the catalytic subunit of cAMP-dependent protein kinase and [ $\gamma\text{-}^{32}\text{P}$ ]ATP-Mg in close correlation with enhancement of enzyme activity. These results confirm our previous results (12) and are further supported by the findings that hormone-sensitive lipase activity was associated with  $^{32}\text{P}$ -labeled protein of  $M_r = 84,000$  after extensive purification of the enzyme from  $^{32}\text{P}$ -labeled intact fat cells (42).

Previous attempts to purify hormone-sensitive lipase (41) have been only partly successful due to interaction of the enzyme with lipid, resulting in large lipid-protein aggregates, and to difficulties encountered when trying to extract the lipids with solvents (45, 46) or detergents. However, with techniques for detergent solubilization of adipose tissue preparations developed in this laboratory and used for purification of monoacylglycerol lipase (18), hormone-sensitive lipase was solubilized and partially purified (12). Recently, extensive purification of hormone-sensitive lipase from hen adipose

tissue was reported by Berglund *et al.* (47). They identified the enzyme with a protein of apparent  $M_r = 42,000$  by SDS-PAGE. Although this finding may reflect a species difference, the characteristics of this purified lipase are so different that its relation to hormone-sensitive lipase must be questioned. The purified enzyme, the predominant protein by SDS-PAGE in the final preparation, had a maximal specific enzyme activity of approximately  $0.1 \mu\text{mol}$  of fatty acid/min/mg of protein, less than 1/1000 of that calculated for the rat enzyme in this report. The total amount of enzyme purified was also quite low (0.033 unit from 275 g of hen adipose tissue as compared with 7 units from 225 g of rat adipose tissue), reportedly (47) partially due to its marked instability. Moreover, although the  $M_r = 42,000$  polypeptide could be phosphorylated by incubation with cAMP-dependent protein kinase and ATP-Mg, enzyme activatability was rapidly and completely lost during the purification. The recent observations by the same workers that the  $M_r$  was 45,000 (47) or, after cautious sample pretreatment, 84,000 by SDS-PAGE (48), may indicate that the purified enzyme represented a proteolytically modified hormone-sensitive lipase.

In the present work, the affinity chromatography accounted for much of the enzyme purification. Although, as discussed above, the mechanism for enzyme binding and elution at this step is not clear, under the conditions used, it was quite reproducible. Interestingly, carboxyhexanoyl-Sepharose substituted with dioctanoin has been used in a similar manner for the purification of a glycerol ester hydrolase of rat intestinal mucosa (36). Also, elution of milk lipoprotein lipase from heparin-substituted agarose gel with detergent has been observed (49). At present we have found no way to continue purification of the small amount of enzyme protein ( $30 \mu\text{g}$  from 200 rats) to homogeneity. Molecular size fractionation has provided little further separation from the remaining protein contaminants.

The specific enzyme activity of the purified hormone-sensitive lipase toward its optimal substrate approached that found with other mammalian tissue lipases such as lipoprotein lipase (50), hepatic (salt-resistant) lipase (51), and monoacylglycerol lipase (18). The DFP inhibition and [ $^3\text{H}$ ]DFP-labeling experiments demonstrated that the molar specific activity with this substrate was in the same order of magnitude as that found with bovine milk lipoprotein lipase (50). The substrate specificity of the enzyme was distinctly different from that of other mammalian tissue lipases (50-52), with much higher relative activity toward diacylglycerol and with activity toward cholesterol esters (Fig. 5). The latter finding probably explains the reported presence of cholesterol esterase activity which could be enhanced with cAMP-dependent protein kinase and ATP-Mg in particulate hormone-sensitive lipase preparations (53) and the recent observation (54) that phenylmethylsulfonyl fluoride inhibited both triacylglycerol and cholesterol ester hydrolase activity to the same extent.

The hallmark of hormone-sensitive lipase is its activatability. In this work, we have shown that the enzyme can be phosphorylated and activated by a cAMP-dependent protein kinase, confirming and extending our previous findings (12). The dependence of activation on phosphorylation demonstrated by their close time relationship clearly indicates the significance of the process. The prompt arrest of phosphorylation by the specific inhibitor protein of cAMP-dependent protein kinase supports the notion of no obligatorily intervening "lipase-kinase" (55), analogous to phosphorylase kinase. There may, however, be other protein kinases, acting independently of cAMP and cAMP-dependent protein kinase, that operate in the regulation of hormone-sensitive lipase.

The physiologic relevance of phosphorylation as a regula-

tory mechanism for adipose tissue lipolysis is supported by the demonstration that the enzyme indeed is phosphorylated in the intact cell (42) and that the degree of phosphorylation closely correlated with the lipolysis rate in cells stimulated with noradrenaline and with insulin (29). However, two discrepancies between the system operating in the intact cell and *in vitro* deserve further comment. In the intact cell, phosphorylation and lipolysis are maximal within minutes following noradrenaline addition. Half-maximal effects after 30 min, obtained in this study, are probably due to the low lipase concentration (approximately  $50 \text{ nM}$ ), with the protein kinase working far below substrate saturation (*cf.* Ref. 56). Assuming that intracellular water is 3%, lipase concentration in adipocytes will be almost  $0.5 \mu\text{M}$ . Since lipases act at oil-water interfaces, the actual concentration may be considerably higher.

Secondly, in the intact cell, phosphorylation of hormone-sensitive lipase increases the rate of lipolysis up to 50-fold, whereas with the isolated enzyme, 2- to 3-fold activation is obtained (*cf.* Ref. 57). Preliminary experiments suggest that this discrepancy is not due to the maximum activity obtained but rather to different basal activities. Intact fat cells from 220-g fed rats released fatty acids, measured by pH-stat titration (24), at rates of  $<0.03$  and  $0.7 \mu\text{mol/min/ml}$  of packed cell volume, under basal conditions and after maximal noradrenaline stimulation, but disruption of the cells by brief sonication (2-3 s) changed these values to 0.24 and 0.35, respectively. Mainly as a result of this almost 10-fold increase of basal lipolytic activity, the activation decreased from more than 20-fold to less than 2-fold. This indicates that the marked hormonal activation of lipolysis in intact fat cells may be mainly due to a very low basal activity, caused, for example, by unfavorable conditions for enzyme-substrate binding which can be overcome by enzyme phosphorylation. Increased binding and activity could also be favored by the dispersion of the big triacylglycerol droplets ( $30\text{--}50 \mu\text{m}$  in diameter) by sonication, which dramatically increases the interfacial area and number of available substrate molecules. Sonicated fat emulsions used for enzyme assay would not provide substrate in the form found in nonactivated fat cells; thus, high basal activity and a low degree of activation should be expected in the cell-free system.

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## REFERENCES

- Belfrage, P., Fredrikson, G., Nilsson, N. O. and Strålfors, P. (1979) *INSERM Colloq.* **87**, 161-178.
- Vaughan, M., and Steinberg, D. (1965) in *Handbook of Physiology* (Renold, A. E., and Cahill, G. F., Jr., eds) Section 5, pp. 239-251, Williams & Wilkins, Baltimore.
- Hollenberg, C. H., Raben, M. S., and Astwood, E. B. (1961) *Endocrinology* **68**, 589-598.
- Vaughan, M., and Steinberg, D. (1963) *J. Lipid Res.* **4**, 193-199.
- Björntorp, P., and Furman, R. H. (1962) *Am. J. Physiol.* **203**, 316-322.
- Rizack, M. A. (1964) *J. Biol. Chem.* **239**, 392-395.
- Vaughan, M., Berger, J. E., and Steinberg, D. (1964) *J. Biol. Chem.* **239**, 401-409.
- Huttunen, J. K., Steinberg, D., and Mayer, S. E. (1970) *Proc. Natl. Acad. Sci. U. S. A.* **67**, 290-295.
- Corbin, J. D., Reimann, E. M., Walsh, D. A., and Krebs, E. G. (1970) *J. Biol. Chem.* **245**, 4849-4851.
- Khoo, J. C., Steinberg, D., Huang, J. J., and Vagelos, P. R. (1976)

- J. Biol. Chem.* **251**, 2882-2890
11. Severson, D. L., Khoo, J. C., and Steinberg, D. (1977) *J. Biol. Chem.* **252**, 1484-1489
  12. Belfrage, P., Jergil, B., Strålfors, P., and Tornqvist, H. (1977) *FEBS Lett.* **17**, 259-264
  13. Tornqvist, H., Krabisch, L., and Belfrage, P. (1974) *J. Lipid Res.* **15**, 291-294
  14. Tornqvist, H., Krabisch, L., and Belfrage, P. (1972) *J. Lipid Res.* **13**, 424-426
  15. Tornqvist, H., Bjorgell, P., Krabisch, L., and Belfrage, P. (1978) *J. Lipid Res.* **19**, 654-656
  16. Nilsson, Å., Nördén, H., and Wilhelmsson, L. (1973) *Biochim. Biophys. Acta* **296**, 593-603
  17. Chang, K.-J., Marcus, N. A., and Cuatrecasas, P. (1974) *J. Biol. Chem.* **249**, 6854-6865
  18. Tornqvist, H., and Belfrage, P. (1976) *J. Biol. Chem.* **251**, 813-819
  19. Nimmo, H. G., and Cohen, P. (1977) *Adv. Cyclic Nucleotide Res.* **8**, 145-266
  20. Corkill, J. M., Goodman, J. F., and Otteville, R. H. (1961) *Trans. Faraday Soc.* **57**, 1627-1636
  21. Helenius, A., McCaslin, D. R., Fries, E., and Tanford, C. (1979) *Methods Enzymol.* **56**, 734-749
  22. Tornqvist, H., Nilsson-Ehle, P., and Belfrage, P. (1978) *Biochim. Biophys. Acta* **530**, 474-486
  23. Nilsson-Ehle, P., Tornqvist, H., and Belfrage, P. (1972) *Clin. Chim. Acta* **42**, 383-390
  24. Nilsson, N. O., and Belfrage, P. (1979) *J. Lipid Res.* **20**, 557-560
  25. Jergil, B., and Ohlsson, R. (1974) *Eur. J. Biochem.* **46**, 13-25
  26. Meisler, M. H., and Langan, T. A. (1969) *J. Biol. Chem.* **244**, 4961-4968
  27. Titanji, V. (1977) *Biochim. Biophys. Acta* **481**, 140-151
  28. Laemmli, U. K. (1970) *Nature (Lond.)* **227**, 680-685
  29. Nilsson, N. O., Strålfors, P., Fredrikson, G., and Belfrage, P. (1980) *FEBS Lett.* **111**, 125-130
  30. Laskey, R. A., and Mills, A. D. (1977) *FEBS Lett.* **82**, 314-316
  31. Chamberlain, J. P. (1979) *Anal. Biochem.* **98**, 132-135
  32. Tornqvist, H., and Belfrage, P. (1976) *J. Lipid Res.* **17**, 542-545
  33. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
  34. Belfrage, P., Wiebe, T., and Lundquist, A. (1970) *Scand. J. Clin. Lab. Invest.* **26**, 53-60
  35. Kirkegaard, L. H. (1976) in *Methods of Protein Separation* (Catsimpoolas, N., ed) Vol. 2, pp. 279-319, Plenum Press, New York
  36. Fernandez-Lopez, V., Serrero, G., Négrel, R., and Ailhaud, G. (1976) *Eur. J. Biochem.* **71**, 259-270
  37. Cohen, J. A., Oosterbaan, R. A., and Berends, F. (1967) *Methods Enzymol.* **11**, 686-702
  38. Helmerhorst, E., and Stokes, G. B. (1980) *Anal. Biochem.* **104**, 130-135
  39. Huttunen, J. K., Ellingboe, J., Pittman, R. C., and Steinberg, D. (1970) *Biochim. Biophys. Acta* **218**, 333-346
  40. Weller, M. (1979) *Protein Phosphorylation*, Pion Ltd., London
  41. Huttunen, J. K., and Steinberg, D. (1971) *Biochim. Biophys. Acta* **239**, 411-427
  42. Belfrage, P., Fredrikson, G., Nilsson, N. Ö., and Strålfors, P. (1980) *FEBS Lett.* **111**, 120-124
  43. Steinberg, D., and Vaughan, M. (1965) in *Handbook of Physiology* (Renold, A. E., and Cahill, G. F., Jr., eds) Section 5, pp. 335-347, Williams & Wilkins, Baltimore
  44. Scow, R. O. (1965) in *Handbook of Physiology* (Renold, A. E., and Cahill, G. F., Jr., eds) Section 5, pp. 437-453, Williams & Wilkins, Baltimore
  45. Strand, O., Vaughan, M., and Steinberg, D. (1964) *J. Lipid Res.* **5**, 554-562
  46. Tsai, S.-C., Belfrage, P., and Vaughan, M. (1970) *J. Lipid Res.* **11**, 466-472
  47. Berglund, L., Khoo, J. C., Jensen, D., and Steinberg, D. (1980) *J. Biol. Chem.* **255**, 5420-5428
  48. Khoo, J. C., Berglund, L., Jensen, D., and Steinberg, D. (1980) *Biochim. Biophys. Acta* **619**, 440-444
  49. Kinnunen, P. K. J., Huttunen, J. K., and Ehnholm, C. (1976) *Biochim. Biophys. Acta* **450**, 342-351
  50. Bengtsson, G. (1979) thesis, Umeå University Medical Dissertation 48, Umeå University
  51. Kuusi, T. (1979) thesis, University of Helsinki
  52. Brockerhoff, H., and Jensen, R. G. (1974) *Lipolytic Enzymes*, Academic Press, New York
  53. Pittman, R. C., and Steinberg, D. (1975) *J. Biol. Chem.* **250**, 4505-4511
  54. Lee, F.-T., and Yeaman, S. J. (1980) *Biochem. Soc. Trans.* **8**, 728-729
  55. Steinberg, D., Mayer, S. E., Khoo, J. C., Müller, E. A., Müller, R. E., Fredholm, B., and Eichner, R. (1975) *Adv. Cyclic Nucleotide Res.* **5**, 549-568
  56. Engstrom, L. (1980) in *Molecular Aspects of Cellular Regulation* (Cohen, P., ed) Vol. 1, pp. 11-31, Elsevier/North-Holland Biomedical Press, Amsterdam
  57. Wise, L. S., and Jungas, R. L. (1978) *J. Biol. Chem.* **253**, 2624-2627



## Positional Specificity of Hormone-sensitive Lipase from Rat Adipose Tissue\*

Gudrun Fredrikson and Per Belfrage

From the Department of Physiological Chemistry, University of Lund, Lund, Sweden

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Hormone-sensitive lipase, purified from rat adipose tissue (Fredrikson, G., Strålfors, P., Nilsson, N. O., and Belfrage, P. (1981) *J. Biol. Chem.* 256, 6311-6320), has been incubated with tri-, di-, and monooleoyl [ $^3\text{H}$ ]glycerol, and the acylglycerol reaction products were isolated by thin layer chromatography on silicic acid, impregnated with boric acid. Trioleoylglycerol was hydrolyzed with the intermediate accumulation of monooleoylglycerol, mainly the 2-isomer, and a small amount of 1,2(2,3)-, but no measurable, 1,3-dioleoylglycerol. 2-Monooleoylglycerol was also the major acylglycerol reaction product from 1,2(2,3)-dioleoylglycerol hydrolysis, which occurred at a  $V_{\text{max}}$  of 60% of that with the 1,3-isomer. Part of the 1(3)-monooleoylglycerols found were formed by acyl migration, but 2-ester bond cleavage was directly demonstrated by the use of 1,3-dioleoyl-2-[ $^{14}\text{C}$ ]oleoyl [ $^3\text{H}$ ]glycerol as substrate, and by determination of the  $^{14}\text{C}/^3\text{H}$  ratios of the acylglycerol reaction products. Based on the hydrolysis of specific monooleoylglycerol isomers, it was estimated that the 1(3)-ester bonds of the acylglycerols were hydrolyzed 3- to 4-fold faster than the 2-ester bonds. The main lipolytic reaction sequence catalyzed by hormone-sensitive lipase is thus triacylglycerol  $\rightarrow$  1,2(2,3)-diacylglycerol  $\rightarrow$  2-monoacylglycerol. However, the preference for the 1(3)-ester bonds is less marked than that of, e.g. pancreatic and lipoprotein lipase.

Most mammalian acylglycerol lipases have a marked preference for the primary ester bonds of their substrates, e.g. pancreatic lipase (1-3), lipoprotein lipase (4-6), hepatic lipase (7, 8), lingual lipase (9), and lysosomal acid lipase (10). In contrast, the bile salt-stimulated lipase (11) and monoacylglycerol lipases (12, 13) have been reported to lack positional specificity. The question has not yet been solved for the hormone-sensitive lipase of adipose tissue, since this enzyme has only recently been obtained in sufficiently purified form, free from other lipases, e.g. lipoprotein lipase and monoacylglycerol lipase (14). In this report, the possible positional specificity of detergent-solubilized and purified hormone-sensitive lipase from rat adipose tissue (14) has been examined. Incubations with labeled di- and monooleoylglycerol isomers, and with trioleoylglycerol labeled with [ $^3\text{H}$ ]glycerol and [ $^{14}\text{C}$ ]

oleic acid in ester position 2, demonstrated also that this enzyme has a relatively high specificity for the 1(3)-ester bonds.

### MATERIALS AND METHODS

**Substrates**—Tri-, di-, and monooleoyl [ $^3\text{H}$ ]glycerol, and 1,3-dioleoyl-2-[ $^{14}\text{C}$ ]oleoylglycerol (all at least 99.3% pure and more than 99% oleic acid) were synthesized and stored as described in Refs. 5 and 15-17. From the racemic mixtures, approximately 97% pure dioleoylglycerol isomers were prepared by column chromatography, and approximately 90% pure 2-monooleoylglycerol by thin layer chromatography on silicic acid, impregnated with boric acid (18). The racemic mixture of monooleoylglycerols (approximately 90% of the 1(3)-isomer) was used without further purification as 1(3)-monooleoylglycerol substrate. Monooleoylglycerols were stable to isomerization for at least 1 month in heptane at  $-80^\circ\text{C}$ .

**Enzyme**—Hormone-sensitive lipase was detergent-solubilized and purified from rat adipose tissue (14) up to and including the hydroxypatite concentration step, to approximately 4% purity. Results were the same when 50% pure enzyme was tested in a few cases, but it was available in insufficient amounts and could not be concentrated to the same extent (14). The enzyme preparations used contained no measurable lipoprotein lipase, monoacylglycerol lipase, or lysosomal acid lipase. They were stored in 0.5 M potassium phosphate, pH 7.0, 50% (w/v) glycerol, 1 mM dithioerythritol, and 2 mM of the nonionic detergent *n*-dodecyloctaoxyethylene ether (Nikko Chemicals, Tokyo, Japan). The detergent was required to keep the amphiphilic enzyme protein solubilized and enzymatically active (14). The homogeneous catalytic subunit of cyclic AMP-dependent protein kinase was prepared from rat adipose tissue (19). Homogeneous porcine pancreatic lipase and colipase were gifts from Dr. Charlotte Erlanson-Albertsson of this department and sodium taurodeoxycholate was a gift from Monika Lindström, also of this department. Pancreatic lipase was diluted in 30 mM potassium phosphate, pH 7.0, 6 mM sodium taurodeoxycholate, in the presence of a 10-fold molar excess of colipase.

**Incubations**—Tri- and dioleoylglycerol substrates were obtained by sonication (20) of 66 or 310  $\mu\text{g}$  of each lipid, respectively, and 40  $\mu\text{g}$  of phospholipids (phosphatidylcholine and -inositol, 3:1 (w/w), see Ref. 14) in 1.0 ml of 0.1 M potassium phosphate, pH 7.0. Defatted bovine serum albumin (21) was added to 5% (tri-oleoylglycerol substrates) or 2% (w/v) final concentration. Monooleoylglycerol substrate (366  $\mu\text{g}$ ) was sonicated with 2 mM *n*-dodecyloctaoxyethylene ether essentially as described in Ref. 17, and was prepared in the same buffer as the other substrates but contained no albumin.

Enzyme and substrate (100  $\mu\text{l}$ ) were incubated in a final volume of 200  $\mu\text{l}$  at  $37^\circ\text{C}$ , with the proper measures taken to ensure that detergent, glycerol, and buffer concentrations in all samples were the same. Phosphorylation of hormone-sensitive lipase with the catalytic subunit of cyclic AMP-dependent protein kinase and ATP-Mg $^{2+}$  was performed as described in Ref. 14.

**Extraction and Separation of Reaction Products**—Glycerol or fatty acids were isolated essentially as described in Refs. 17 and 22, and acylglycerols extracted as described in Refs. 5 and 23. A suitable aliquot of redissolved lipids was chromatographed in acetone:chloroform (12:88) on a silicic acid thin layer plate (Kieselgel 60, Merck, West Germany), which had been soaked in 0.4 M boric acid, and activated at  $110^\circ\text{C}$  for 2 h before use. The lipid spots were visualized in iodine vapor, the corresponding part of the thin layer plate cut out, and the radioactivity measured by liquid scintillation after addition of 1 ml of methanol:H $_2$ O (1:1), and 10 ml of Insta-Gel

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(Packard):toluene (1:1). The recovery of labeled lipids over the entire procedure was more than 90%.

### RESULTS

**Hydrolysis of Trioleoylglycerol**—2-Monooleoylglycerol was the major acylglycerol reaction product (up to 65%) from trioleoylglycerol hydrolysis, while little 1,2(2,3)- (<7%), and no measurable 1,3-dioleoylglycerol (<15% of the diacylglycerols) was found (Fig. 1). The proportion of the 2-monoacylglycerol increased with shorter incubation times (Fig. 1, inset).

Phosphorylation of the enzyme by cyclic AMP-dependent protein kinase and ATP-Mg<sup>2+</sup>, which is known to activate it towards a triacylglycerol substrate (14) but with little effect on the activity towards partial acylglycerols (24, 25), did not appreciably change the reaction product pattern compared to a nonphosphorylated control (data not presented).

A large part of the 1(3)-monooleoylglycerol in the incubations (Fig. 1), was likely to have been the result of time-dependent isomerization of the 2-isomer, since the equilibrium mixture of monoacylglycerols is 90% 1(3)-isomer. It was observed that such isomerization was rapid during the incubation conditions used. In labeled 2-monooleoylglycerol substrate, the contents of the 1(3)-isomer increased from approximately 15 to 30% after 5 min, and 50% after 10 min. This isomerization was related to the presence of albumin, since in 2-monooleoylglycerol prepared with detergent (see "MATERIALS AND METHODS"), the contents of 1(3)-isomer did not increase appreciably (from 14 to 17% in 10 min).

**Hydrolysis of Dioleoylglycerol**—Hormone-sensitive lipase is intrinsically much more active toward dioleoylglycerol, and detergent inhibition is less pronounced with a di- than with a trioleoylglycerol substrate (14, 20). The products of hydrolysis of 1,2(2,3)-dioleoyl-[<sup>3</sup>H]glycerol could therefore be examined after more extensive hydrolysis and at shorter incubation times than was possible with the trioleoylglycerol substrate. Also, the hydrolysis of this dioleoylglycerol substrate proceeded with an accumulation of monooleoylglycerol, which constituted up to 90% (Fig. 2, a and b) of the products. Most of this (up to 70%) was the 2-isomer. Also, in this case the accumulation was more pronounced at shorter incubation times (Fig. 2b).

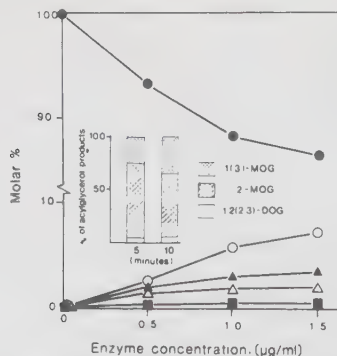


FIG. 1. Molar composition of the reaction mixture after incubation of trioleoylglycerol with different concentrations of hormone-sensitive lipase. Trioleoyl [<sup>3</sup>H]glycerol was incubated for 10 min with the enzyme at the indicated concentrations. Inset, relative composition of the acylglycerol reaction products with 1.0 µg/ml of enzyme after different incubation times. Values are means of duplicates. ●, trioleoyl [<sup>3</sup>H]glycerol; ■, 1,2(2,3)-dioleoyl [<sup>3</sup>H]glycerol (1,2(2,3)-DOG); ▲, 2-monooleoyl [<sup>3</sup>H]glycerol (2-MOG); △, 1(3)-monooleoyl [<sup>3</sup>H]glycerol (1(3)-MOG); ○, [<sup>3</sup>H]glycerol.

The dioleoylglycerol isomers were hydrolyzed at different rates by the lipase: 1,3-dioleoylglycerol 1.6 times  $\pm$  0.07 (mean  $\pm$  S.E.,  $n = 7$ ) faster at substrate saturation than 1,2(2,3)-dioleoylglycerol. Only the maximal reaction rates were different; the apparent  $K_m$  (determined from double reciprocal plots) was similar for both isomers (approximately 33 µM).

**Hydrolysis of Monooleoylglycerol**—The activity of the enzyme toward 1(3)-monooleoylglycerol was 3 times ( $3.0 \pm 0.26$ ,  $n = 3$ ) that toward the 2-isomer (Fig. 3). This difference was approximately the same at different substrate concentrations. True substrate saturation was not obtained, possibly due to interaction of the detergent in the substrate with the lipase. Part, or most, of the measured hydrolysis of the 2-monooleoylglycerol substrate could have been due to hydrolysis of the 1(3)-isomer present (10%). In a mixture containing considerably more 1(3)-isomer (65%), both isomers were hydrolyzed, the 1(3)-isomer approximately 4 times faster than the 2-isomer (results not illustrated). Since with these substrates no appreciable isomerization occurred (see above), this value is likely to correctly reflect the difference in the rates of hydrolysis.

**Hydrolysis of 1,3-Dioleoyl-2-[<sup>14</sup>C]oleoyl [<sup>3</sup>H]glycerol**—The results above indicated preference of the hormone-sensitive lipase for the 1(3)-ester bond of the acylglycerols. The occurrence of 1(3)-monoacylglycerols, and the relatively rapid production of glycerol (Figs. 1 and 2), was not unexpected because

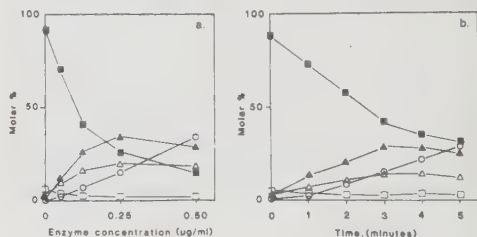


FIG. 2. Molar composition of the reaction mixture after incubation of 1,2(2,3)-dioleoylglycerol with hormone-sensitive lipase. a, enzyme at indicated concentrations was incubated with substrate for 5 min. b, enzyme (0.2 µg/ml) was incubated with substrate for the indicated times. Values are means of duplicates. □, 1,3-dioleoyl [<sup>3</sup>H]glycerol; other symbols as in Fig. 1.

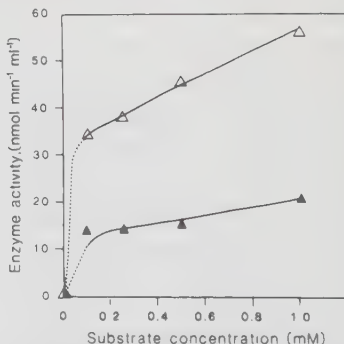


FIG. 3. Hydrolysis of monooleoylglycerol isomers by hormone-sensitive lipase. Enzyme (20 ng/ml) was incubated with 1(3)- and 2-monooleoyl [<sup>3</sup>H]glycerol substrates at the indicated concentrations for 15 min. Values are means of triplicates. Symbols as in Fig. 1.



TABLE I

$^{14}\text{C}/^3\text{H}$  ratios in the acylglycerol reaction products isolated after incubation of hormone-sensitive and pancreatic lipase with 1,3-dioleoyl-2- $^{14}\text{C}$ oleoyl $^3\text{H}$ glycerol substrate

The substrate was incubated with the respective lipases (hormone-sensitive lipase, 2.5  $\mu\text{g}/\text{ml}$ , and pancreatic lipase, 5  $\mu\text{g}/\text{ml}$ ) for 10 min.

| Acylglycerol species | Pancreatic lipase <sup>a</sup>   |                            | Hormone-sensitive lipase <sup>b</sup> |                            |
|----------------------|----------------------------------|----------------------------|---------------------------------------|----------------------------|
|                      | % acylglycerol reaction products | $^{14}\text{C}/^3\text{H}$ | % acylglycerol reaction products      | $^{14}\text{C}/^3\text{H}$ |
| 1,3-Di-              | 4.9 $\pm$ 1.8                    | 0.98 $\pm$ 0.12            | <1                                    | Not measurable             |
| 1,2(2,3)-Di-         | 31.5 $\pm$ 2.1                   | 1.04 $\pm$ 0.03            | 9.6 $\pm$ 2.4                         | 1.16 $\pm$ 0.10            |
| 1(3)-Mono-           | 18.1 $\pm$ 2.2                   | 0.97 $\pm$ 0.04            | 33.6 $\pm$ 1.0                        | 0.36 $\pm$ 0.04            |
| 2-Mono-              | 45.4 $\pm$ 1.2                   | 1.02 $\pm$ 0.01            | 56.8 $\pm$ 1.9                        | 0.80 $\pm$ 0.02            |

<sup>a</sup> Values are mean  $\pm$  S.E.,  $n = 3$ .

<sup>b</sup>  $n = 5$ .

of the rapid isomerization of monoacylglycerols observed under the experimental conditions (see above). To examine the extent of the isomerization, trioleoylglycerol, labeled with  $^{14}\text{C}$  in the fatty acid in position 2, and with  $^3\text{H}$  in the glycerol moiety, was used as substrate, as previously done with other lipases (5, 6, 23, 26). Pancreatic lipase was incubated with the same substrate, under closely similar conditions, in order to compare the results with those obtained with a lipase which has an absolute specificity for the 1(3)-ester bonds (1-3). Amounts of enzymes were adjusted so that similar rates of monoacylglycerol production were obtained.

The  $^{14}\text{C}/^3\text{H}$  ratio of the trioleoylglycerol substrate (set to 1.00) did not change appreciably during its hydrolysis, which averaged 7% with pancreatic lipase and 5% with hormone-sensitive lipase. Under these experimental conditions, the incubation with pancreatic lipase resulted in the formation of some 1,3-dioleoylglycerol (13% of diacylglycerols), and 1(3)-monooleoylglycerol (28% of the monoacylglycerols) (Table I). As demonstrated by the  $^{14}\text{C}/^3\text{H}$  ratios of about unity, these reaction products were formed almost entirely by acyl migration within the 1,2(2,3)di- and the 2-monoacylglycerol isomers. The results were thus entirely compatible with an enzyme exhibiting an almost absolute specificity for the primary ester bonds (*cf.* 5, 6, 26).

Less diacylglycerol accumulated in the incubations with the hormone-sensitive lipase, as could be expected from the relatively higher activity of this enzyme towards diacylglycerol (14), as compared to pancreatic lipase (27). The amounts of monoacylglycerols were similar to those obtained with pancreatic lipase as was the proportion of the 1(3)-isomer (37%). However, a striking finding was the much lower  $^{14}\text{C}/^3\text{H}$  ratio in this acylglycerol reaction product (0.36 compared to 0.97), about half of that of the corresponding 2-isomer. This shows that a considerable part of the 1(3)-monooleoylglycerol must have been formed by direct cleavage of the 2-ester bond of the 1,2(2,3)dioleoylglycerol (which would lead to a 1(3)-monoacylglycerol with a ratio of 0). The lower than unity ratio for the 2-monoacylglycerol fraction (0.80) is likely to be the result of acyl migration within some of the 1(3)-isomers produced by such direct 2-ester cleavage.

#### DISCUSSION

Hormone-sensitive lipase has a marked preference for the primary ester bonds of the acylglycerol substrates because (a) no measurable 1,3-dioleoylglycerol was found during triacylglycerol hydrolysis, (b) this isomer was hydrolyzed considerably faster than the 1,2(2,3)-diacylglycerol, (c) 2-monoacylglycerol was the major acylglycerol product during tri- as well as

diacylglycerol hydrolysis, and (d) it was hydrolyzed at a 3- to 4-fold lower rate than the 1(3)-isomer.

Although most of the 1(3)-monoacylglycerol and glycerol formation during the incubations is likely to have been the result of isomerization (see above), the results with the doubly-labeled trioleoylglycerol showed that some 1(3)-monoacylglycerol must also have been produced by hydrolysis of the 2-ester bond of diacylglycerols. This was evident from the direct comparison with pancreatic lipase, incubated with the same substrate under very similar conditions. Thus, hormone-sensitive lipase differs from pancreatic lipase, and also from lipoprotein lipase (4-6) in having a less absolute preference for the primary ester bonds of the acylglycerol substrates. The main course of the lipolytic reaction catalyzed by hormone-sensitive lipase is, nevertheless, the same as for these enzymes: triacylglycerol  $\rightarrow$  1,2(2,3)-diacylglycerol  $\rightarrow$  2-monoacylglycerol.

We have previously reported that the relative maximal activities of hormone-sensitive lipase towards tri-, di-, and monooleoylglycerol are 1:10:4 (14). The dioleoylglycerol used in those experiments was mainly the 1,2(2,3)-isomer, and the monooleoylglycerol was almost entirely (>90%) the 1(3)-isomer.<sup>1</sup> On the basis of the results of the present work, the main substrates likely to be found in the fat cell during lipolysis catalyzed by hormone-sensitive lipase would be tri-, 1,2(2,3)-di-, and 2-monoacylglycerol. The relative maximal rates of hydrolysis of these substrates can thus be estimated to be approximately 1:10:1. After phosphorylation/activation of the enzyme (usually 2.5-fold towards triacylglycerol (14)), the corresponding values would be approximately 1:4:0.4.

Accumulation of monoacylglycerol during hormone-sensitive lipase-catalyzed hydrolysis of tri- and diacylglycerol substrates *in vitro*, has been reported previously (14, 28). Based on the above calculations, monoacylglycerol hydrolysis could be expected to be rate-limiting, at least during hormone-stimulated lipolysis *in vivo*, and 2-monoacylglycerol could be expected to accumulate. It has been found, however, that monoacylglycerol does not appreciably accumulate during hormone-stimulated lipolysis (29, 30), although some increase of diacylglycerol concentration has been reported (29, 30). This points strongly to an important role for the specific monoacylglycerol lipase in the fat cell, known to hydrolyze both monoacylglycerol isomers at equal rates (13).

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#### REFERENCES

- Mattson, F. H., Benedict, J. H., Martin, J. B., and Beck, L. W. (1952) *J. Nutr.* **48**, 335-344.
- Mattson, F. H., and Beck, L. W. (1955) *J. Biol. Chem.* **214**, 115-125.
- Borgström, B. (1964) *J. Lipid Res.* **5**, 522-531.
- Borgström, B., and Carlson, L. A. (1957) *Biochim. Biophys. Acta* **24**, 638-639.
- Nilsson-Ehle, P., Belfrage, P., and Borgström, B. (1971) *Biochim. Biophys. Acta* **248**, 114-120.
- Nilsson-Ehle, P., Egelrud, T., Belfrage, P., Olivecrona, T., and Borgström, B. (1973) *J. Biol. Chem.* **248**, 6734-6737.
- Assmann, G., Krauss, R. M., Fredrickson, D. S., and Levy, R. I. (1973) *J. Biol. Chem.* **248**, 7184-7190.
- Åkesson, B., Gronowitz, S., and Herslöf, B. (1976) *FEBS Lett.* **71**, 241-244.
- Paltauf, F., Esfandi, F., and Holasek, A. (1974) *FEBS Lett.* **40**, 119-123.
- Warner, T. G., Dambach, L. M., Shin, J. H., and O'Brien, J. S. (1981) *J. Biol. Chem.* **256**, 2952-2957.
- Hernell, O., and Blackberg, L. (1982) *Pediatr. Res.* **16**, 882-885.

<sup>1</sup> G. Fredrikson and P. Belfrage, unpublished results.

12. Pope, J. L., McPherson, J. C., and Tidwell, H. C. (1966) *J. Biol. Chem.* **241**, 2306-2310
13. Tornqvist, H., and Belfrage, P. (1976) *J. Biol. Chem.* **251**, 813-819
14. Fredrikson, G., Strålfors, P., Nilsson, N. Ö., and Belfrage, P. (1981) *J. Biol. Chem.* **256**, 6311-6320
15. Morgan, R. G. H., and Borgström, B. (1969) *Q. J. Exp. Physiol.* **54**, 228-243
16. Krabisch, L., and Borgström, B. (1965) *J. Lipid Res.* **6**, 156-157
17. Tornqvist, H., Krabisch, L., and Belfrage, P. (1974) *J. Lipid Res.* **15**, 291-294
18. Thomas, A. E., III, Sharoun, J. E., and Ralston, H. (1965) *J. Am. Oil-Chem. Soc.* **42**, 789-792
19. Strålfors, P., and Belfrage, P. (1982) *Biochim. Biophys. Acta* **721**, 434-440
20. Fredrikson, G., Strålfors, P., Nilsson, N. Ö., and Belfrage, P. (1981) *Methods Enzymol.* **71**, 636-646
21. Chen, R. F. (1967) *J. Biol. Chem.* **242**, 173-181
22. Belfrage, P., and Vaughan, M. (1969) *J. Lipid Res.* **10**, 341-344
23. Noma, A., and Borgström, B. (1971) *Biochim. Biophys. Acta* **227**, 106-115
24. Heller, R. A., and Steinberg, D. (1972) *Biochim. Biophys. Acta* **270**, 65-73
25. Belfrage, P., Jergil, B., Strålfors, P., and Tornqvist, H. (1977) *FEBS Lett.* **75**, 259-264
26. Nilsson-Ehle, P., Garfinkel, A. S., and Schotz, M. C. (1974) *Lipids* **9**, 548-553
27. Brockerhoff, H., and Jensen, R. G. (1974) *Lipolytic Enzymes*, pp. 34-90, Academic Press, New York
28. Berglund, L., Khoo, J. C., Jensen, D., and Steinberg, D. (1980) *J. Biol. Chem.* **255**, 5420-5428
29. Scow, R. O., Stricker, F. A., Pick, T. Y., and Clary, T. R. (1965) *Ann. N. Y. Acad. Sci.* **131**, 288-301
30. Arner, P., and Ostman, J. (1974) *Biochim. Biophys. Acta* **369**, 209-221

HORMONE-SENSITIVE LIPASE FROM SWINE ADIPOSE TISSUE:  
Identification and Some Properties

by

Fook-Thean Lee and Stephen J. Yeaman  
Department of Biochemistry, Ridley Building, The University,  
Newcastle upon Tyne NE1 7RU, UK

and

Gudrun Fredrikson, Peter Strålfors and Per Belfrage  
Department of Physiological Chemistry 4, University of Lund,  
S-220 07 Lund, Sweden

Running title: Swine hormone-sensitive lipase

Address for Correspondence and Proofs:

Dr. S.J. Yeaman, Department of Biochemistry, Ridley Building, The  
University, Newcastle upon Tyne NE1 7RU, U.K. (Tel. 0632 328511  
Ext. 3433)

Abstract - 1. Swine adipose tissue hormone-sensitive lipase, purified 475-fold to 10% protein purity, has been identified as a polypeptide of  $M_r=84,000$ . 2. The enzyme has high specific activity against tri-, di- and monoacylglycerols, as well as cholesterol esters, and is inhibited by millimolar NaF, and micromolar  $HgCl_2$  and DFP. 3. The enzyme polypeptide serves as a substrate for cyclic AMP-dependent protein kinase. 4. The characteristics of the hormone-sensitive lipase from swine adipose tissue are similar to those reported previously for the enzyme from rat. 5. They differ from those reported for the lipase from chicken adipose tissue, and possible reasons for these differences are discussed.

Foot-note 1

Abbreviations: DFP, diisopropylfluorophosphate; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis;  $C_{13}E_{12}$ ,  $C_{12}E_8$ ,  $C_8E_6$ , alkyl polyoxyethylene ether detergents with the general formula  $C_nH_{2n+1}(OCH_2CH_2)_xOH$  abbreviated to  $C_nE_x$  (see Fredrikson et al., 1981).

## INTRODUCTION

The rate-limiting enzyme in lipolysis in adipose tissue is hormone-sensitive lipase. This enzyme is under acute hormonal control, being activated by catecholamines and deactivated by insulin (Steinberg, 1976; Strålfors and Belfrage, 1984). Although data were available already 20 years ago (Rizack, 1964) which suggested that regulation was mediated by reversible phosphorylation, progress in this area was hindered for many years by lack of knowledge of the molecular properties of the enzyme, and it is only relatively recently that direct evidence has been obtained for this mechanism (Strålfors and Belfrage, 1984). Identification of the enzyme polypeptide is an essential pre-requisite to studying the phosphorylation of the enzyme both in vitro and in vivo. Belfrage et al. (1977) first identified the enzyme from rat adipose tissue as a polypeptide of subunit  $M_r=84,000$ . Subsequently, the lipase has been extensively purified and several of its molecular properties (Fredrikson et al., 1981) as well as the mechanism for the hormonal control of its activity (Strålfors and Belfrage, 1983; Strålfors et al., 1984) have been characterized. In addition to catalysing the hydrolysis of triacylglycerol, the enzyme is also active against di- and monoacylglycerol and cholesterol esters. The enzyme has a high specific activity (approximately 400  $\mu\text{mol}$  fatty acid produced per min per mg protein using dioleoylglycerol as substrate) (Strålfors and Belfrage 1983) and from this it can be calculated that the levels of enzyme protein in the cell correspond to only 1-2  $\mu\text{g}$  per g of rat adipose tissue (Fredrikson et al., 1981).

In contrast, lipase purified from chicken adipose tissue has been reported by Berglund et al. (1980) as having a subunit molecular weight of 42,000 and a low specific activity of approximately 0.1  $\mu\text{mol}$  fatty acid produced from dioleoylglycerol per min per mg protein. Using the same purification procedures



these workers found that the enzyme from rat adipose tissue had a subunit  $M_r=84,000$  (Khoo et al., 1980). Inter-species variation has been postulated as the explanation for the different observed subunit  $M_r$  of the rat and chicken enzymes (Berglund et al., 1980).

We have studied hormone-sensitive lipase from swine adipose tissue as part of an attempt to prepare the enzyme from a more easily available enzyme source than rat adipose tissue. The swine may be suitable also from the aspect that the adrenergic control of the lipolysis in its adipose tissue has recently been carefully characterized (Mersman, 1984a,b). The finding, reported below, that the enzyme from this species is a polypeptide of  $M_r=84,000$  with properties very similar to those reported for the enzyme from rat is discussed in relation to possible inter-species differences in the molecular properties of the lipase.

#### MATERIALS AND METHODS

Materials: Sources of substrates, protease inhibitors, detergents and other reagents were as described in (Fredrikson et al., 1981; Fredrikson et al., 1982). The catalytic subunit of cAMP-dependent protein kinase was purified as previously described (Strålfors and Belfrage, 1982).

Enzyme Purification: Mesenteric adipose tissue was obtained from pigs (Sus domesticus, Swedish Landrace), weighing approx. 70 kg from a local abattoir and the hormone-sensitive lipase was purified using the methods previously described (Fredrikson et al., 1981) for the purification of hormone-sensitive lipase from rat adipose tissue. The enzyme in the pH 5.2 precipitate fraction was solubilised by sonication in 6.5% (w/v) of the non-ionic detergent  $C_{13}E_{12}$  (Footnote 1) in buffer and subjected to two successive gradient sievortptive chromatographies on QAE-Sephadex

A-25. Enzyme was concentrated by reversible adsorption on hydroxylapatite and high activity fractions were subjected to affinity chromatography on lipid-containing Ultrogel AcA 34 in 0.8% (w/v) C<sub>8</sub>E<sub>6</sub> (Fredrikson *et al.*, 1981). Enzyme was again concentrated by hydroxylapatite chromatography and C<sub>8</sub>E<sub>6</sub> was exchanged for C<sub>12</sub>E<sub>8</sub>. The final enzyme was obtained in 0.5 M potassium phosphate, pH 7.0, 50% (w/v) glycerol, 1 mM dithioerythritol and 0.1% (w/v) C<sub>12</sub>E<sub>8</sub>.

Enzyme Assays: Hormone-sensitive lipase was assayed against the various substrates as in (Fredrikson *et al.*, 1981), except that 60 mM potassium phosphate pH 7.0 was used as buffer. One unit of enzyme activity releases 1  $\mu$ mol of fatty acid/min at 37°C and specific activity is expressed as units/mg protein.

Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulphate: SDS-PAGE was performed as described in (Laemmli, 1970) with modification (Nilsson *et al.*, 1980). Proteins were precipitated by 9% (w/v) trichloroacetic acid in 30% (v/v) acetone and extracted with solvent before electrophoresis. Reference proteins were glycogen phosphorylase ( $M_r$ =97,400), transferrin ( $M_r$ =76,700), bovine serum albumin ( $M_r$ =67,000), catalase ( $M_r$ =57,500) and ovalbumin ( $M_r$ =43,000). Autoradiography, fluorography and densitometric scanning were carried out as described in (Fredrikson *et al.*, 1981).

Phosphorylation and [<sup>3</sup>H]DFP-Labeling of Hormone-Sensitive Lipase: The final enzyme preparation, in 5 mM imidazole-HCl pH 7.0, containing 1 mM dithioerythritol, 0.1M NaCl, 50% (w/v) glycerol, 2 mM C<sub>12</sub>E<sub>8</sub>, 0.05% (w/v) bovine serum albumin was incubated at 30° for 30 min in the presence of catalytic subunit of cyclic AMP-dependent protein kinase and 5 mM MgCl<sub>2</sub>, 0.1 mM [ $\gamma$ -<sup>32</sup>P]ATP (10<sup>3</sup> cpm/pmol). Phosphorylation was terminated by precipitation of the protein for analysis by SDS-PAGE. For

labelling with [ $^3\text{H}$ ]DFP, the final enzyme preparation was incubated with 19  $\mu\text{M}$  [ $^3\text{H}$ ]DFP ( $14 \times 10^3$  dpm/pmol) for 30 min at  $37^\circ$  and the protein was then precipitated for analysis by SDS-PAGE.

Other Methods: Protein was either monitored by absorbance at 280 nm or determined with a scaled down version (Tornqvist and Belfrage, 1976) of the method of Lowry et al. (Lowry et al., 1954) after trichloroacetic acid precipitation in detergent followed by extraction with diethyl ether: ethanol, 3:1 (v/v). Bovine serum albumin was used as standard. Concentration of sodium chloride was measured by its conductivity.

## RESULTS

Using methods developed for the purification of hormone-sensitive lipase from rat (Fredrikson et al., 1981), the enzyme from swine adipose tissue was purified 475-fold. The chromatographic properties of the enzymes from the two sources were similar except that the swine lipase appeared slightly more acidic as judged by the requirement for higher concentrations of NaCl (55 mM as against 40 mM) for elution from QAE-Sephadex. The final product had a specific activity of 21 U/mg using a dioleoylglycerol analogue as substrate (Fredrikson et al., 1981) and had significant activity also against triacylglycerol, monoacylglycerol and cholesterol esters. The relative maximal activity against tri-, di-, monooleoylglycerol and cholesterol oleate was 1:12.7:5.6:1.6. The corresponding values for the enzyme from rat are 1:10:4:1.5 (Fredrikson et al., 1981).

The activity of swine hormone-sensitive lipase was inhibited by certain selective inhibitors namely  $\text{HgCl}_2$ , NaF and DFP, each of which acts in a different manner. Fifty percent inhibition of the activity against the dioleoylglycerol analogue was observed

in the presence of 10  $\mu$ M, 4 mM and 7.5  $\mu$ M of the respective compounds. Again those inhibition properties are closely related to those reported for the rat enzyme (Fredrikson et al., 1981).

In order to identify the swine hormone-sensitive lipase protein the experiments illustrated in Fig. 1 were performed. Protein staining of an SDS polyacrylamide slab gel of the purest lipase preparation showed several polypeptide bands (Fig. 1a, right lane). One of these polypeptides, labelled HSL in the Figure, with  $M_r=84,000$  and constituting approx. 10% of the stainable protein, was enriched approximately 10-fold during the final affinity chromatography step of the purification procedure (compare Fig. 1a, right with left lane). (The major, phosphorylatable polypeptide with  $M_r=123,000$  (Fig. 1a,b) was ATP-citrate lyase (P. Strålfors, unpublished)). The  $M_r=84,000$  polypeptide was  $^{32}$ P-phosphorylated when incubated with [ $\gamma$ - $^{32}$ P]ATP-Mg $^{2+}$  and a homogenous preparation of the catalytic subunit of cyclic AMP-dependent protein kinase (Fig. 1b, right lane). Besides ATP-citrate lyase (see above) the only other significant  $^{32}$ P-phosphorylation occurred in a polypeptide with an  $M_r=77,000$  (Fig. 1b, right lane). Both the  $M_r=84,000$  and the  $M_r=77,000$  [ $^{32}$ P]phosphopeptides were enriched during the final purification step. [ $^3$ H]DFP-labelling was used to establish the identity of the lipase protein. The stability of the covalent bond formed with this radiolabelled inhibitor allows identification of the lipase by fluorography of the  $^3$ H-labelled proteins fractionated by SDS-PAGE. When the most highly-purified swine hormone-sensitive lipase preparation was incubated with [ $^3$ H]DFP the  $M_r=84,000$  polypeptide was, almost selectively, radio-labelled (Fig. 1c, right lane). The  $M_r=84,000$  species was the only protein interacting with DFP that was enriched during the final affinity chromatography step. Especially, no detectable [ $^3$ H]DFP was found in the  $M_r=77,000$  phosphorylatable protein species.

On the basis of these experiments we conclude that the  $M_r=84,000$  polypeptide is the hormone-sensitive lipase protein from swine adipose tissue. For comparison the position of a  $^{32}\text{P}$ -phosphorylated rat hormone-sensitive lipase on an SDS polyacrylamide slab gel is shown in Fig. 1d. No difference between the  $M_r$  of the respective enzyme species can be observed.

## DISCUSSION

We report here purification of swine adipose tissue hormone-sensitive lipase and identify the enzyme as a polypeptide of  $M_r=84,000$ . The final product had a specific activity of 21 U/mg and the lipase constituted 10-11% of the total protein, indicating that the specific activity of homogeneous enzyme is over 200 U/mg. These characteristics are very similar to those reported previously for hormone-sensitive lipase from rat adipose tissue (Fredrikson et al., 1981) and for the hormone-sensitive cholesterol ester hydrolase purified from bovine adrenal cortex (Cook et al., 1982) and corpus luteum (Cook et al., 1983). This enzyme catalyses a rate-limiting reaction in steroidogenesis and has been shown to be identical or very similar to the adipose tissue hormone-sensitive lipase (Cook et al., 1982).

As discussed above Berglund et al. (1980), working with chicken adipose tissue, have purified the lipase 500-fold and identified the enzyme as a polypeptide of  $M_r=42,000$ . The specific activity of the final product of the chicken lipase purification was exceedingly low, being several thousand-fold lower than reported for the highly-purified lipase from rat (Fredrikson et al., 1981). These differences have been attributed to species differences (Berglund et al., 1981; Khoo and Steinberg, 1984) but in view of the fact that the enzyme has now been shown to be very similar in tissues of rat (Fredrikson et al., 1981), cattle (Cook et al., 1982; 1983) and swine (present work) this seems increas-



ingly unlikely, especially in view of the low specific activity of the chicken enzyme. A more probable explanation is that the  $M_r=42,000$  polypeptide present in the preparation of chicken lipase was a major contaminant and that the lipase polypeptide was present in undetectable amounts. Interestingly, partially-purified preparations of the enzyme from bovine adrenal cortex contain a major phosphorylatable protein contaminant of  $M_r=41,000$  which during further purification can be completely resolved from the lipase/esterase activity (Cook et al., 1981).

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## REFERENCES

- Belfrage, P., Jergil, B., Strålfors, P. & Tornqvist, H. (1977) Hormone-sensitive lipase of rat adipose tissue: identification and some properties of the enzyme protein. FEBS Lett. 17, 259-264
- Berglund, L., Khoo, J.C., Jensen, D. & Steinberg, D. (1980) Resolution of hormone-sensitive triglyceride/diglyceride lipase from monoglyceride lipase of chicken adipose tissue. J. Biol. Chem. 255, 5420-5428.
- Cook, K.G., Lee, F.-T. & Yeaman, S.J. (1981) Hormone-sensitive cholesterol ester hydrolase of bovine adrenal cortex. Identification of the enzyme protein. FEBS Lett. 132, 10-14.
- Cook, K.G., Yeaman, S.J., Strålfors, P., Fredrikson, G. & Belfrage, P. (1982) Direct evidence that adrenal cortex cholesterol ester hydrolase is the same enzyme as adipose tissue hormone-sensitive lipase. Eur. J. Biochem. 125, 245-249.
- Cook, K.G., Colbran, R.J., Snee, J. & Yeaman, S.J. (1983) Cytosolic cholesterol ester hydrolase from bovine corpus luteum, its purification, identification and relationship to hormone-sensitive lipase. Biochim. Biophys. Acta 752, 46-53.
- Fredrikson, G., Strålfors, P., Nilsson, N.Ö. & Belfrage, P. (1981) Hormone-sensitive lipase of rat adipose tissue. Purification and some properties. J. Biol. Chem. 256, 6311-6320.
- Fredrikson, G., Krabisch, L. & Belfrage, P. (1982) Synthesis of <sup>14</sup>C-labelled alkyl polyoxyethylene detergents. J. Lipid Res. 23, 1246-1248.
- Khoo, J.C., Berglund, L., Jensen, D. & Steinberg, D. (1980) Hormone-sensitive lipase of rat adipose tissue: correlation of activity with a protein of molecular weight 84,000. Biochim. Biophys. Acta 619, 440-444.
- Khoo, J.C. & Steinberg, D. (1983) Hormone-sensitive lipase of adipose tissue. In P.D. Boyer (ed.) The Enzymes, Vol. XVI, Academic Press, New York, pp. 183-204.

- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227,680-685.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.
- Mersman, H.J. (1984a) Specificity of  $\beta$ -adrenergic control of lipolysis in swine adipose tissue. Comp. Biochem. Physiol. 77C, 39-42.
- Mersman, H.J. (1984b) Adrenergic control of lipolysis in swine adipose tissue. Comp. Biochem. Physiol. 77C, 43-53.
- Nilsson, N.Ö., Strålfors, P., Fredrikson, G. & Belfrage, P. (1980) Effects of noradrenaline and insulin on phosphorylation of hormone-sensitive lipase and on lipolysis in intact rat adipocytes. FEBS Lett. 111, 125-130.
- Rizack, M.A. (1964) Activation of an epinephrine-sensitive lipolytic activity from adipose tissue by adenosine 3',5'-phosphate. J. Biol. Chem. 239, 392-395.
- Steinberg, D. (1976) Interconvertible enzymes in adipose tissue regulated by cyclic AMP-dependent protein kinase. In P. Greengard and G.A. Robison (eds.), Advances in Cyclic Nucleotide Research, Vol. 7, Raven Press, New York, pp 157-198.
- Strålfors, P. & Belfrage, P. (1982) Properties and purification of the catalytic subunit of cyclic AMP-dependent protein kinase of adipose tissue. Biochim. Biophys. Acta 721,434-440.
- Strålfors, P. & Belfrage, P. (1983) Phosphorylation of hormone-sensitive lipase by cyclic AMP-dependent protein kinase. J. Biol. Chem. 285, 15146-15152.
- Strålfors, P. & Belfrage, P. (1984) Reversible phosphorylation of hormone-sensitive lipase/cholesterol ester hydrolase in the hormonal control of adipose tissue lipolysis and of adrenal steroidogenesis. In P. Cohen (ed.) Enzyme Regulation by Reversible Phosphorylation-Further Advances, Elsevier/North Holland, Amsterdam, pp. 27-62.

Strålfors, P., Björgell, P. & Belfrage, P. (1984) Hormonal regulation of hormone-sensitive lipase in intact adipocytes: identification of phosphorylated sites and effects on the phosphorylation by lipolytic hormones and insulin. Proc. Natl. Acad. Sci, U.S.A. In press.

Tornqvist, H. & Belfrage, P. (1976) Determination of protein in adipose tissue extracts. J. Lipid Res. 17, 542-545.

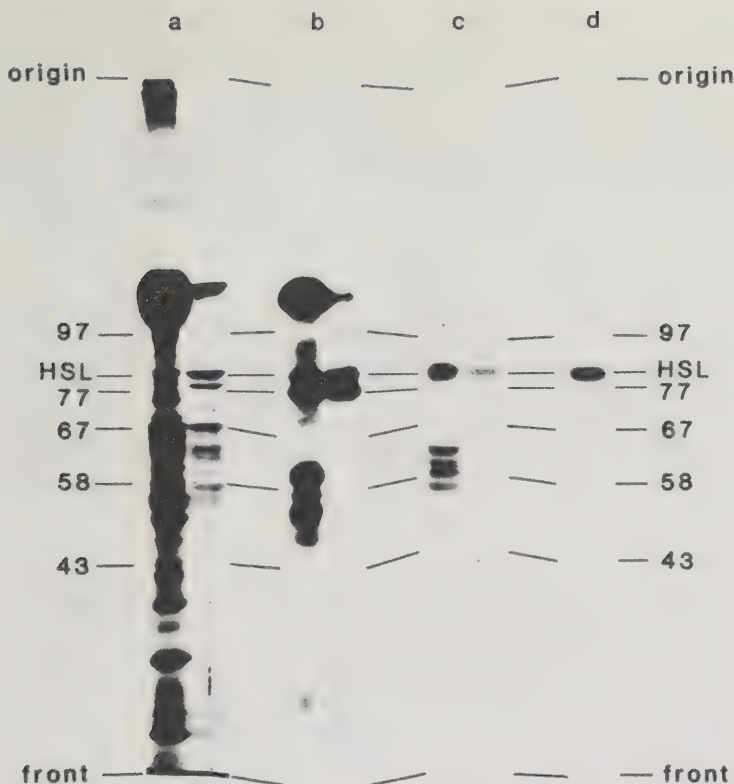
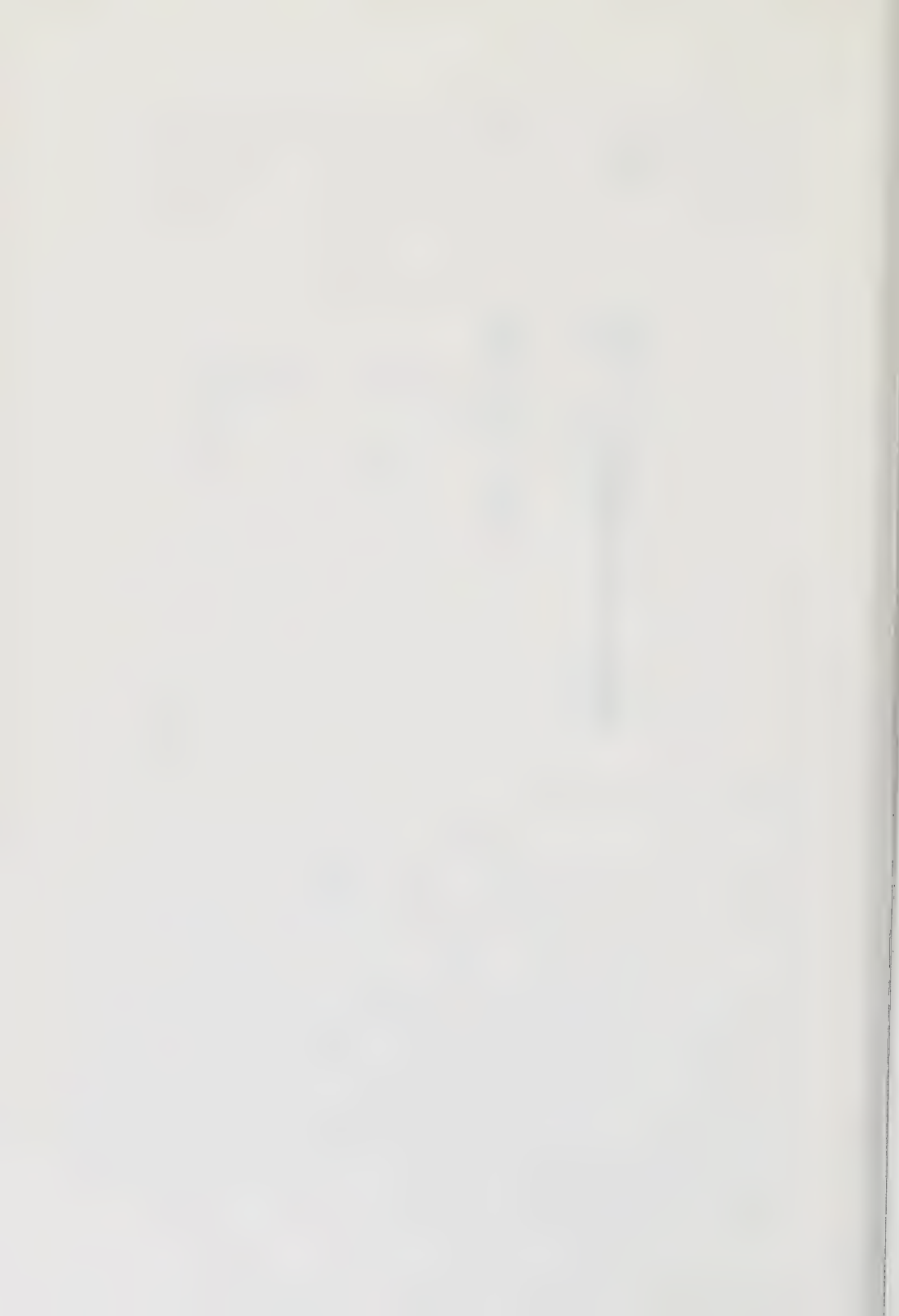


Figure 1: Analysis by SDS-PAGE of Purified Hormone-Sensitive Lipase

Samples were treated as described under Materials and Methods. a. Coomassie Blue stained gel, b. autoradiograph of sample phosphorylated using [ $\gamma$ -<sup>32</sup>P]ATP and c. fluorograph following labelling with [<sup>3</sup>H]DFP. Left lanes: hormone-sensitive lipase peak fractions after second QAE-Sephadex chromatography and concentration on hydroxylapatite; right lanes: after final affinity chromatography on Ultrogel AcA 34. For comparison, d. shows an autoradiograph of rat hormone-sensitive lipase, purified as described in (Fredrikson *et al.*, 1981) up to and including the first hydroxylapatite chromatography (cf. Materials and Methods) and phosphorylated (Strålfors and Belfrage, 1983) with [ $\gamma$ -<sup>32</sup>P]ATP. The  $M_r$  (x 10<sup>-3</sup>) of reference proteins are indicated.





ROLES OF HORMONE-SENSITIVE LIPASE AND MONOACYLGLYCEROL LIPASE IN  
ADIPOSE TISSUE LIPOLYSIS

Gudrun Fredrikson<sup>a</sup>, Hans Tornqvist<sup>b</sup> and Per Belfrage<sup>a</sup>

<sup>a</sup>Department of Physiological Chemistry, University of Lund, Lund,  
Sweden

<sup>b</sup>Department of Pediatrics, University Hospital, University of  
Lund, Lund, Sweden

Correspondence to: G. Fredrikson

Dept. of Physiol. Chem. 4

P.O.Box 750

S-220 07 Lund

Sweden

Foot-note 1

Alkyl polyoxyethylene ether detergents with the general formula  $C_nH_{2n+1}(OCH_2CH_2)_xOH$ , abbreviated to  $C_nE_x$ , were used.  $C_{12}E_8$  (Nikko Chemicals, Tokyo, Japan) was homogeneous, and  $C_{13}E_{12}$  (Berol Kemi AB, Stenungsund, Sweden) was heterogeneous.

Foot-note 2

The abbreviation used is: pH 5.2 ppt, pH 5.2 precipitate fraction.

## SUMMARY

The role of monoacylglycerol lipase in adipose tissue lipolysis has been investigated using different enzyme preparations. First, a crude adipose tissue preparation, containing both monoacylglycerol lipase and hormone-sensitive lipase in approximately the same proportions as in the intact tissue, hydrolyzed emulsified tri- or dioleoyl [ $^3\text{H}$ ]glycerol to completion, with no appreciable accumulation of di- or monooleoylglycerol. Immunoprecipitation of the monoacylglycerol lipase in this tissue preparation considerably decreased glycerol release, indicating accumulation of monoacylglycerols.

Isolated hormone-sensitive lipase hydrolyzed acylglycerols with a marked accumulation of monoacylglycerol in line with the positional specificity of this enzyme (Fredrikson, G. and Belfrage, P. (1983) J. Biol. Chem. 258, 14253-14256). Addition of isolated monoacylglycerol lipase to incubations of purified hormone-sensitive lipase with tri- or dioleoyl [ $^3\text{H}$ ]glycerol, lead to a substantial increase in glycerol release, due to hydrolysis of the accumulated monoacylglycerols. When the relative proportion of the two lipases was similar to that in the intact tissue hydrolysis was complete.

The findings indicate the following roles of the two lipases in adipose tissue lipolysis: hormone-sensitive lipases hydrolyzes triacylglycerol, in the rate-limiting step, and the diacylglycerol products, whereas monoacylglycerol lipase completes lipolysis by hydrolyzing monoacylglycerol.

## INTRODUCTION

Hormone-sensitive lipase of rat adipose tissue has been shown to have a marked preference for the primary ester bonds of its acylglycerol substrates (1). Monoacylglycerol (mainly of the 2-configuration) accumulated when isolated hormone-sensitive lipase was incubated with tri- or diacylglycerol (1,2). However, there is no appreciable accumulation of monoacylglycerol in the intact tissue during adipose tissue lipolysis (3,4). The tissue contains a monoacylglycerol lipase, with high activity towards monoacylglycerol, which lacks positional specificity (5). This lipase has previously been suggested to play a role for completing the hydrolysis of adipose tissue triacylglycerol (1,6,7). Both hormone-sensitive lipase and monoacylglycerol lipase have been solubilized and purified (2,5), and an antiserum has been raised against monoacylglycerol lipase in rabbits (this report), allowing a more detailed study of the process of lipolysis in vitro to be performed. In this work we have used this antiserum to specifically inhibit the monoacylglycerol lipase of crude adipose tissue preparations during hydrolysis of tri- and diacylglycerol substrates. We have also studied the roles of the lipases with various proportions of the isolated enzymes. The results demonstrate that monoacylglycerol lipase is required for complete hydrolysis of the acylglycerol substrates.

## MATERIALS AND METHODS

Substrates. Tri- and dioleoyl[ $^3\text{H}$ ]glycerol and tri- and di[ $^3\text{H}$ ]oleoylglycerol (all > 99% pure and > 99% oleic acid) were synthesized, purified and stored as previously described (1,8-10). 1,2(2,3)-Dioleoylglycerol isomer was separated from 1,3-dioleoylglycerol by column chromatography resulting in approximately 90% pure isomers. The dioleoylglycerol was stable with respect to isomerization for at least one month in dry benzene at +4°C.



Monoacylglycerol lipase was assayed with a micellar monooleoyl-[<sup>3</sup>H]glycerol substrate at pH 8.0 (11), and hormone-sensitive lipase with emulsified mono[<sup>3</sup>H]oleoylmonooleylglycerol at pH 7.0 (12), to follow the activity of the two lipases after immunoprecipitation (see Results).

Enzymes. Hormone-sensitive lipase from rat adipose tissue was solubilized with non-ionic detergent and partially purified as described in (2), up to and including the hydroxyapatite concentration step, to approximately 4% protein purity. The enzyme preparation contained no measurable lipoprotein lipase, monoacylglycerol lipase, or lysosomal acid lipase (2). It was stored in 0.5 M potassium phosphate, pH 7.0, 50% (w/v) glycerol, 1 mM dithioerythritol and 2 mM C<sub>12</sub>E<sub>8</sub>, a non-ionic detergent of the alkyl polyoxyethylene ether type (Foot-note 1), at -80°C. Monoacylglycerol lipase was purified as described earlier (5,13) to more than 90% protein purity and was free from other lipolytic activities. In some experiments a crude adipose tissue preparation was used, the pH 5.2 ppt (Foot-note 2) (2,14). This was prepared by homogenization of adipose tissue and centrifugation at 100,000 x g for 1 h. The fat-depleted infranatant was precipitated at pH 5.2 and the precipitate suspended in 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM dithioerythritol, 10 µg/ml leupeptin, 1 µg/ml pepstatin (protease inhibitors were from the Peptide Institute, Inc., Osaka, Japan). The proportion between the two lipase activities in the pH 5.2 ppt can be assumed to be approximately the same as that in the tissue, since enzyme activity losses during the preparation steps were very similar for the two enzymes (2,5). Both lipases were found in large lipid-protein aggregates (2,5). One ml of the 5.2 ppt contained material from approx. 10 g of rat adipose tissue. Amounts of the enzymes were calculated from their respective specific activities (5,15).

Incubations. Tri- and dioleoylglycerol substrates were prepared by sonication of 66 or 310  $\mu$ g, respectively, of the lipids and 40  $\mu$ g phospholipids (phosphatidylcholine: phosphatidylinositol 3:1 (w/v)) in 1.0 ml 0.1 M potassium phosphate, pH 7.0. Bovine serum albumin, defatted as in (16), was added to a final concentration of 5% for trioleoylglycerol substrate, and 2% (w/v) for dioleoylglycerol.

Equal volumes of enzyme(s) (diluted if necessary with 20 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM dithioerythritol, 0.2 mg/ml bovine serum albumin) and substrate were incubated at 37°C.

Extraction and separation of reaction products. Glycerol or fatty acids produced during the incubations were extracted and measured as previously described (11,17). Analysis of all reaction products, including acylglycerols, was performed after solvent extraction, and separation of the products by thin layer chromatography on boric acid impregnated silicic acid thin layer plates (1,8,18).

Monoacylglycerol lipase antiserum. An antiserum against monoacylglycerol lipase was raised by immunization of a rabbit with the purest preparation of the enzyme protein (>95% homogeneity on sodium dodecylsulphate polyacrylamide gel electrophoresis). Enzyme protein (25  $\mu$ g) in 1 ml of 50% (w/v) sucrose, 1% (w/v) Ampholine, 1 mM EDTA, 1 mM dithioerythritol and 0.2% (w/v) C<sub>13</sub>E<sub>12</sub> was emulsified with 1 ml of Freund's complete adjuvans. One ml of the emulsion was injected subcutaneously into the neck of a rabbit and 1 ml in multiple intracutaneous injections in the back. After 2 weeks, the rabbit was boosted with another 25  $\mu$ g of enzyme protein, injected as above. Antiserum was collected 6 weeks after the first injection and used without further purification. Serum from the same rabbit collected before immunization,

termed preimmune serum, was used in controls. The monoacylglycerol lipase antiserum produced one precipitation line against the purified enzyme preparation after counter immunoelectrophoresis (19) (Fig. 1). The location of the precipitate was demonstrated after treatment with the immunoglobulin fraction of swine antiserum to rabbit immunoglobulins, labelled with horseradish peroxidase (Dakopatts, Copenhagen) (20). The precipitate contained active enzyme since soaking the fresh gel in a solution of p-nitrophenyl acetate (0.5 mg/ml, in 20 mM Tris-HCl, pH 7.4, 1 mM dithioerythritol, 1 mM EDTA and 0.2% (w/v) C<sub>13</sub>E<sub>12</sub>) resulted in selective hydrolysis of the substrate in the precipitate.

## RESULTS

Incubations with the crude tissue preparation: Hydrolysis of trioleoyl[<sup>3</sup>H]glycerol by the crude adipose tissue preparation, the pH 5.2 ppt, was complete (Fig. 2a), di- and monooleoylglycerol isomers never constituting more than 0.5 molar% each. Hormone-sensitive lipase has a higher activity towards dioleoylglycerol than towards trioleoylglycerol (2) and this substrate was also used since a more extensive hydrolysis could be obtained even at short incubation times (Fig. 2b). Under these conditions some monooleoyl[<sup>3</sup>H]glycerol was detected among the reaction products. However, the fact that the hydrolysis of both substrates was found to proceed without any substantial accumulation of monoacylglycerol (the highest values found were 7 molar% 2-monooleoyl[<sup>3</sup>H]glycerol and 4 molar% 1(3)-monooleoyl[<sup>3</sup>H]glycerol after 8 minutes) indicated that monoacylglycerol lipase might be of importance for completing the hydrolysis.

Specific immunoprecipitation of monoacylglycerol lipase was used to inhibit this lipase. The pH 5.2 ppt was incubated with the antiserum towards monoacylglycerol lipase (Fig. 3) and goat anti-rabbit serum was used to separate antibody-bound protein

from unbound. In controls, the pH 5.2 ppt was incubated with preimmune rabbit serum (Fig. 3). Release of glycerol from a trioleoylglycerol substrate was considerably decreased after treatment with antiserum (Fig. 3a). Fatty acid release was also decreased, but not to the same extent (Fig. 3b). Release of glycerol and fatty acids from a dioleoylglycerol substrate was affected in the same way (Fig. 3c,d), but the higher activity of hormone-sensitive lipase towards this substrate (2) made the effects more pronounced. Under the incubation conditions in Fig. 3 approx. 95% of the monoacylglycerol lipase activity was removed from the supernatant with antiserum, as compared to approx. 30% of the activity in controls, in the latter case presumably due to inactivation. Hormone-sensitive lipase activity was inactivated to the same extent (approx. 30% inactivation) in both immune and preimmune serum incubations. The inhibition of hormone-sensitive lipase activity was thus not likely to be due to cross-reactivity. Release of products (Fig. 3a,b) could, in all cases, be inhibited to 90% by incubation in the presence of 100 mM NaF, a hormone-sensitive lipase inhibitor (2).

Incubations with purified enzymes. To further examine the role of monoacylglycerol lipase in lipolysis the purified enzymes were used. Additional evidence for an important role of monoacylglycerol lipase was found when hormone-sensitive lipase was incubated with tri- or dioleoyl[ $^3\text{H}$ ]glycerol. Addition of monoacylglycerol lipase, in an amount chosen so that the two lipases were in approximately the same proportions as they could be calculated to have in the tissue, led to a considerable increase in glycerol release (Fig. 4a,b). This glycerol release resulted from accumulated monooleoylglycerol as confirmed by analysis of all labelled reaction products (Fig. 5). Increasing the amount of monoacylglycerol lipase lead to decreased monooleoyl[ $^3\text{H}$ ]glycerol and increased [ $^3\text{H}$ ]glycerol. When the ratio of monoacylglycerol lipase

to hormone-sensitive lipase approached the same proportion as in the crude tissue preparation, virtually no monooleoylglycerol could be detected (Fig. 5).

## DISCUSSION

The findings of this paper demonstrate that monoacylglycerol lipase has an important role for completing the hydrolysis of triacylglycerol by hormone-sensitive lipase, in vitro. When hormone-sensitive lipase alone was incubated with triacylglycerol monoacylglycerols accumulated in the incubations and such accumulation was not observed when monoacylglycerol lipase was present in the incubations.

Is monoacylglycerol lipase also required for the complete hydrolysis observed in vivo? Determinations of partial acylglycerols in adipose tissue, have shown that the concentration of monoacylglycerol is very low and that it does not increase appreciably after stimulation of lipolysis (3,4,21). In this report, the accumulation of monoacylglycerol in incubations of hormone-sensitive lipase with triacylglycerol was abolished when the two lipases were added in about the same proportion as they have been calculated to have in the tissue. In fact, even after addition of much less monoacylglycerol lipase, most of the accumulated monoacylglycerols were hydrolyzed. This probably indicates that the amount of monoacylglycerol lipase in adipose tissue is high enough to hydrolyze also the higher amounts of monoacylglycerols formed during maximally stimulated lipolysis, which in vitro corresponds to a 2-3-fold stimulation of hormone-sensitive lipase activity (2,15). The latter enzyme is rate-controlling in adipose tissue lipolysis, the hydrolysis of triacylglycerol being the rate-limiting step (22). The hydrolysis of diacylglycerol by hormone-sensitive lipase should rarely become rate-limiting, in view of the high activity of hormone-sensitive lipase towards



this substrate (2), even if an increase in the concentration of diacylglycerol in adipose tissue has sometimes been reported (4,21). This probably reflects a change from one steady-state concentration to another when lipolysis is stimulated (for further discussion see ref. 22). The above considerations indicate that monoacylglycerol lipase also completes lipolysis in the intact adipose tissue by hydrolyzing monoacylglycerols formed.

Hormone-sensitive lipase, or a closely similar enzyme, has recently been identified in steroid-producing tissues (23,24) and may also be present in several other tissues (for discussion, see ref. 22). Monoacylglycerol lipase also seems to have a wide tissue distribution (H. Tornqvist and K. Musil, unpublished results). Monoacylglycerol lipase may thus have a general role for completing intracellular lipolysis of triacylglycerols in animal cells.

The enzyme has also been proposed to have a role in the final degradation step of chylomicron triacylglycerols (7). Lipoprotein lipase, which catalyzes the first steps of this reaction, has a positional specificity for the 1(3)-ester bonds, leaving 2-monoacylglycerols as a reaction product (25,26). It has been shown that these monoacylglycerols are hydrolyzed before resynthesis of triacylglycerol in adipose tissue (27). A platelet monoacylglycerol lipase has been proposed to account for this hydrolysis (28), but, since it has been shown that monoacylglycerols actually reach the adipocyte (29), the intracellular monoacylglycerol lipase is more likely to be responsible for the hydrolysis.

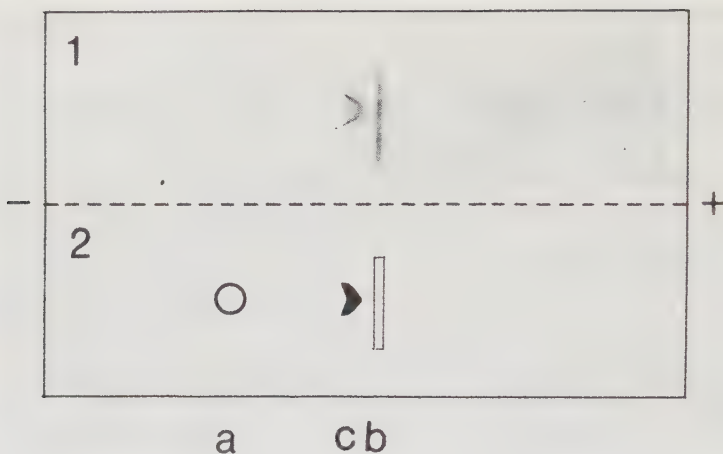
## ACKNOWLEDGEMENT

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## REFERENCES

1. Fredrikson, G. and Belfrage, P. (1983) J. Biol. Chem. 258, 14253-14256
2. Fredrikson, G., Strålfors, P., Nilsson, N.Ö. and Belfrage, P. (1981) J. Biol. Chem. 256, 6311-6320
3. Vaughan, M. and Steinberg, D. (1963) J. Lipid Res. 4, 193-199
4. Scow, R.O. (1965) in Handbook of Physiology (Renold, A.E. and Cahill, G.F., Jr., eds.) Section 5, pp. 437-453, Williams & Wilkins, Baltimore.
5. Tornqvist, H. and Belfrage, P. (1976) J. Biol. Chem. 251, 813-819
6. Vaughan, M., Berger, J.E. and Steinberg, D. (1964) J. Biol. Chem. 239, 401-409
7. Tornqvist, H., Nilsson-Ehle, P. and Belfrage, P. (1978) Biochim. Biophys. Acta 530, 474-486
8. Nilsson-Ehle, P., Belfrage, P. and Borgström, B. (1971) Biochim. Biophys. Acta 248, 114-120
9. Morgan, R.G.H. and Borgström, B. (1969) Q. J. Exp. Physiol. 54, 228-243
10. Krabisch, L. and Borgström, B. (1965) J. Lipid Res. 6, 156-157
11. Tornqvist, H., Krabisch, L. and Belfrage, P. (1974) J. Lipid Res. 15, 291-294
12. Tornqvist, H., Björgell, P., Krabisch, L. and Belfrage, P. (1978) J. Lipid Res. 19, 654-656
13. Tornqvist, H. and Belfrage P. (1981) Methods Enzymol. 71, 646-652
14. Huttunen, J.K., Ellingboe, J., Pittman, R.C. and Steinberg, D. (1970) Biochim. Biophys. Acta 218, 333-346
15. Strålfors, P. and Belfrage, P. (1983) J. Biol. Chem. 258 15146-15152
16. Chen, R.F. (1967) J. Biol. Chem. 242, 173-181

17. Belfrage, P. and Vaughan, M. (1969) J. Lipid Res. 10, 341-344
18. Noma, A. and Borgström, B. (1971) Biochim. Biophys. Acta 227, 106-115
19. Wadström, T. (1983) Scand. J. Immunology 17 Suppl. 10, 97-102
20. Kjaervig Broe, M. and Ingild, A. (1983) Scand. J. Immunol. 17 Suppl. 10, 255-258
21. Arner, P. and Östman, J. (1974) Biochim. Biophys. Acta 369, 209-221
22. Belfrage, P., Fredrikson, G., Strålfors, P. and Tornqvist, H. (1984) in Lipases (Borgström, B. and Brockman, H., eds.) pp. 365-416. Elsevier Scientific Publishing Co., Amsterdam.
23. Cook, K.G., Yeaman, S.J., Strålfors, P., Fredrikson, G. and Belfrage, P. (1982) Eur. J. Biochem. 125, 245-249
24. Cook, K.G., Colbran, R.J., Snee, J. and Yeaman, S.J. (1983) Biochim. Biophys. Acta 752, 46-53
25. Borgström, B. and Carlsson, L.A. (1957) Biochim. Biophys. Acta 24 638-639
26. Nilsson-Ehle, P., Egelrud, T., Belfrage, P., Olivecrona, T. and Borgström, B. (1973) J. Biol. Chem. 248, 6734-6737
27. Olivecrona, T. and Belfrage, P. (1965) Biochim. Biophys. Acta 98, 81-93
28. Bry, K., Kuusi, T., Andersson, L.C. and Kinnunen, P.K.J. (1979) FEBS Lett. 106, 111-114
29. Scow, R.O., Blanchette-Mackie, E.J. and Smith, L.C. (1976) Circulation Research 39, 149-162



**Figure 1.** Counter immunoelectrophoresis of purified monoacylglycerol lipase against its antiserum. (1): Visualization of the precipitates using peroxidase-labelled swine-antirabbit serum. (2): Drawing shows sites of application of (A) 120 ng monoacylglycerol lipase in 4  $\mu$ l and (B) 6  $\mu$ l of antiserum, and (C) monoacylglycerol lipase activity indicated by p-nitrophenol (yellow colour) after hydrolysis of p-nitrophenyl acetate added to the fresh gel (Materials and Methods). The 0.8% (w/v) agarose gel contained 0.2% (w/v)  $C_{13}E_{12}$  in 20 mM barbital buffer, pH 8.6. The electrophoresis was run for 30 min at 21°C.



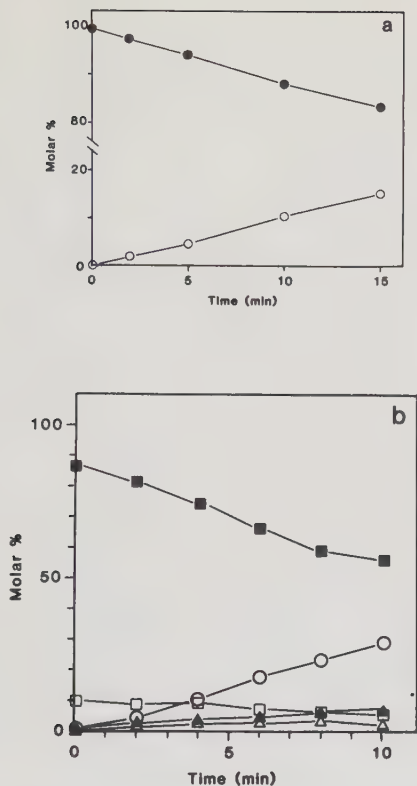


Figure 2. Reaction products after hydrolysis of trioleoyl[<sup>3</sup>H]glycerol and 1,2(2,3)-dioleoyl[<sup>3</sup>H]glycerol by a crude adipose tissue fraction. Trioleoyl[<sup>3</sup>H]glycerol (a) and 1,2(2,3)-dioleoyl[<sup>3</sup>H]glycerol (b) substrates were incubated with pH 5.2 ppt, corresponding to 6 ng monoacylglycerol lipase and 3 ng hormone-sensitive lipase (a) or 1 ng and 0.5 ng (b) of the respective enzymes. Trioleoyl[<sup>3</sup>H]glycerol ● ; 1,3-dioleoyl[<sup>3</sup>H]glycerol □ ; 1,2(2,3)-dioleoyl[<sup>3</sup>H]glycerol ■ ; 2-monooleoyl[<sup>3</sup>H]glycerol ▲ ; 1(3)-monooleoyl[<sup>3</sup>H]glycerol △ ; [<sup>3</sup>H]glycerol ○ . In a 1,3- and 1,2(2,3)-di-, and 1(3)- and 2-monooleoyl[<sup>3</sup>H]glycerol were below 0.5 molar%. Values are means of duplicates.

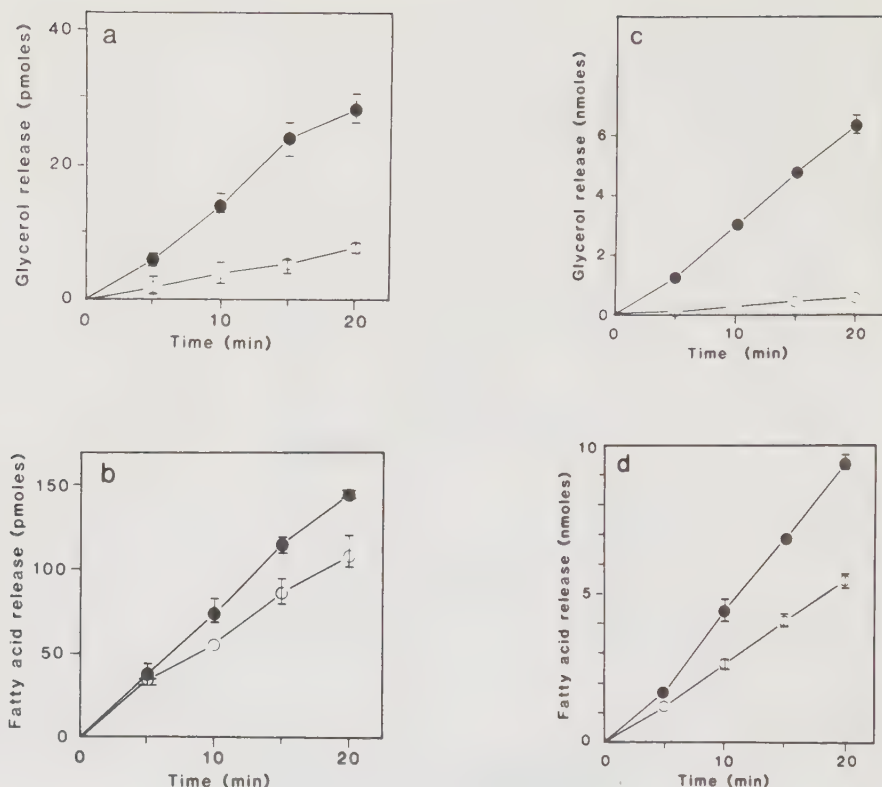


Figure 3. Release of fatty acids and glycerol by a crude adipose tissue fraction after immunoprecipitation of monoacylglycerol lipase. pH 5.2 ppt (200  $\mu$ l) was incubated with antiserum (○) or normal rabbit serum (●) diluted 2-fold (200  $\mu$ l) for 30 min at 40°C. Goat antirabbit serum (500  $\mu$ l) was then added and the incubation continued for another 30 min at 40°C. The precipitate was collected by centrifugation. Samples of the supernatant were assayed towards trioleoyl[ $^3$ H]glycerol (a), tri[ $^3$ H]oleoylglycerol (b), 1,2(2,3)-dioleoyl[ $^3$ H]glycerol (c) and 1,2(2,3)-di[ $^3$ H]oleoylglycerol (d) for the indicated incubation times. Results are means of triplicates. Range is indicated as a vertical bar, unless covered by the symbol.

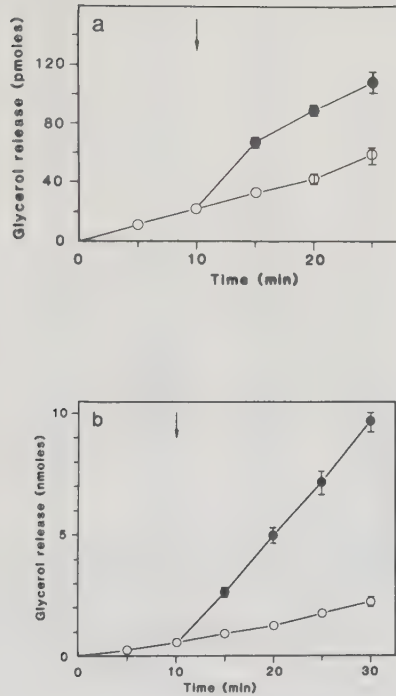
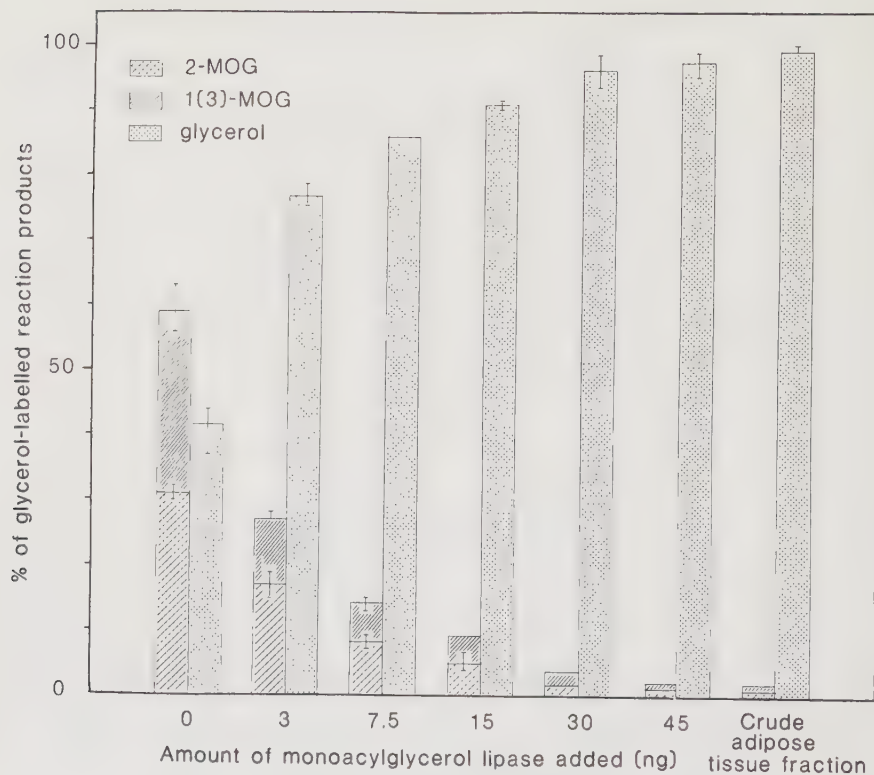


Figure 4. Glycerol release from trioleoylglycerol and dioleoylglycerol after incubation with purified hormone-sensitive lipase, and effect of addition of purified monoacylglycerol lipase on this release. Purified hormone-sensitive lipase, 5 ng (a), or 1 ng (b), was incubated with trioleoyl[ $^3\text{H}$ ]glycerol (a) and 1,2(2,3)-dioleoyl[ $^3\text{H}$ ]glycerol (b) for the indicated times in 6 parallel incubations. After 10 min of incubation monoacylglycerol lipase, 15 ng (a) or 1.5 ng (b), was added (as indicated by arrows) in 3 of 6 incubations and the incubations were continued. Filled symbols indicate values obtained after addition of monoacylglycerol lipase. Range is indicated by a vertical bar, unless covered by the symbols ( $n=6$  for 5 and 10 min,  $n=3$  for 15-30 min).



**Figure 5.** Reaction products after hydrolysis of trioleoyl [ $^3\text{H}$ ]glycerol by hormone-sensitive lipase alone, or with added monoacylglycerol lipase. Hormone-sensitive lipase (45 ng) was incubated with the indicated amounts of monoacylglycerol lipase for 10 min at 37°C. The amount of monoacylglycerol lipase enzyme protein was kept constant in the incubations by addition of denatured (100°C, 10 min) monoacylglycerol lipase, to a total amount of 45 ng enzyme protein in each incubation. The result of a parallel incubation with pH 5.2 ppt, in an amount giving the same total hydrolysis of substrate, is shown as a reference. 2-MOG, 2-monooleoylglycerol; 1(3)-MOG, 1(3)-monooleoylglycerol. The results are means of triplicates, range is indicated by a vertical bar.

## Direct Evidence that Cholesterol Ester Hydrolase from Adrenal Cortex is the Same Enzyme as Hormone-Sensitive Lipase from Adipose Tissue

Kenneth G. COOK and Stephen J. YEAMAN

Department of Biochemistry, University of Newcastle upon Tyne

Peter STRÄLFORS, Gudrun FREDRIKSON, and Per BELFRAGE

Department of Physiological Chemistry 4, University of Lund

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Several properties of the cytosolic cholesterol ester hydrolase from bovine adrenal cortex were investigated and those properties were compared directly with those of the well-characterised hormone-sensitive lipase, the rate-limiting enzyme in adipose tissue lipolysis. Properties examined included: (a) activity against different substrates; (b) susceptibility to inhibition by NaF,  $Hg^{2+}$  ions and diisopropyl fluorophosphonate; (c) subunit molecular weight as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate; (d) ability to serve as a substrate for cyclic AMP-dependent protein kinase; (e) effect of phosphorylation on enzyme activity; and (f) degradation pattern of polypeptides following limited proteolysis.

In all respects the two enzymes exhibited essentially identical characteristics. It is therefore concluded that the same protein, or two very similar proteins, catalyses the hydrolysis of cholesterol esters in adrenal cortex and lipolysis in adipose tissue.

The implication of this finding is discussed in relation to the hormonal control of steroidogenesis in adrenal cortex and of lipolysis in adipose tissue.

Cholesterol ester hydrolase catalyses one of the rate-limiting steps in steroidogenesis in the adrenal cortex [1]. This enzyme catalyses the release of free cholesterol from stores of cholesterol esters present as lipid droplets in the cytoplasm. The free cholesterol is then transported into the mitochondria where it is converted to pregnenolone by the side-chain cleavage enzyme system. Following stress induced by ether anaesthesia, which is known to elevate the circulating levels of corticotrophin, the cholesterol ester stores are depleted and the activity of the cholesterol ester hydrolase is elevated [2]. Corticotrophin is thought to exert most of its effects via the actions of cyclic AMP, and it has been shown that, in the high-speed supernatant from adrenal cortex, the cholesterol ester hydrolase becomes activated following incubation with cyclic AMP and Mg-ATP [3], suggesting that activation results from phosphorylation of the enzyme.

In addition to cholesterol esters, the lipid droplets contain significant levels of phospholipids and triacylglycerol, and adrenal cortex contains a triacylglycerol hydrolase which also becomes activated in response to corticotrophin [4]. This has led to the suggestion that one enzyme may catalyse both the cholesterol ester and triacylglycerol hydrolase activities and indeed the two activities co-purify through a limited purification procedure [5]. The ratio of the two activities does vary, however, in fractions obtained by precipitation at different concentrations of ammonium sulphate and the cholesterol ester hydrolase is more susceptible to inhibition by active

site-directed organophosphates [5]. This can be at least partly explained by the finding that adrenal cortex contains a second distinct triacylglycerol hydrolase which has low activity against cholesterol esters and which is not inhibited by phenylmethylsulphonyl fluoride, another active-site-directed compound [6].

Hormone-sensitive lipase in adipose tissue hydrolyses both triacylglycerol and cholesterol esters and is activated by phosphorylation by cyclic-AMP-dependent protein kinase [7,8]. This hormone-sensitive lipase, which catalyses the rate-limiting step in lipolysis, has been purified from rat adipose tissue to a state approaching homogeneity and many of its molecular and catalytic properties have been investigated [8,9].

The cholesterol ester hydrolase from bovine adrenal cortex has recently been purified and the enzyme protein identified [10]. Several of the properties of this enzyme are similar to those of the adipose tissue hormone-sensitive lipase. Both have a subunit  $M_r$  of 84000, both are active against cholesterol esters and triacylglycerol and both enzymes are inactivated by treatment with DFP. This suggests that one and the same or two similar enzymes may catalyse both hormonally-stimulated lipolysis in adipose tissue and the production of free cholesterol in adrenal cortex, a process also under hormonal control.

In this communication we compare directly the molecular and catalytic properties of rat adipose tissue hormone-sensitive lipase and cholesterol ester hydrolase from bovine adrenal cortex. It is concluded that the two enzymes are very similar, if not identical. In addition, we demonstrate that the adrenal cortex enzyme can be phosphorylated by the catalytic subunit of cyclic-AMP-dependent protein kinase and that phosphorylation leads to activation of the enzyme.

**Abbreviations.** DFP, diisopropylfluorophosphonate; corticotrophin, adrenocorticotrophic hormone; QAE-Sephadex, quaternary diethyl-(2-hydroxypropyl)aminoethyl – Sephadex.

**Enzymes.** Cholesterol esterase or cholesterol ester hydrolase (EC 3.1.1.13); triacylglycerol lipase or hormone-sensitive lipase (EC 3.1.1.3); cyclic-AMP-dependent protein kinase (EC 2.7.1.37).



## MATERIALS AND METHODS

1(3)-Mono[ $^3\text{H}$ ]oleoyl-2-O-oleylglycerol (monooleoylalkylglycerol) and cholesterol [ $^3\text{H}$ ]oleate were synthesised as described previously [11,12]. 1,3-di[ $^3\text{H}$ ]oleoyl-2-O-oleylglycerol (dioleoylalkylglycerol) was synthesised by the same method as monooleoylalkylglycerol [11] using an excess of the acylchloride. Alkylpolyoxyethylene ether detergent, homogeneous  $\text{C}_{12}\text{E}_8$  [ $\text{C}_{12}\text{H}_{25}(\text{OCH}_2\text{CH}_2)_8\text{OH}$ ], was obtained from Nikko Chemicals (Tokyo, Japan) and was treated as in [8]. *Staphylococcus aureus* V8 protease was from Miles Laboratories. [ $^3\text{H}$ ]DFP was from Amersham International Ltd. [ $\gamma$ - $^{32}\text{P}$ ]ATP was synthesised as in [13].

### Enzyme Preparations

Rat adipose tissue hormone-sensitive lipase was purified as described in [8], through the QAE-Sephadex chromatographies and concentrated on hydroxyapatite. The enzyme was obtained in 0.5 M potassium phosphate, pH 7.0, 50% (w/v) glycerol, 1 mM dithioerythritol and 2 mM  $\text{C}_{12}\text{E}_8$ . Adrenal cortex cholesterol ester hydrolase was partially purified, in the presence of Triton X-100, as described previously [10]. The final material was dialysed against 5 mM potassium phosphate, pH 7.0, 50% (w/v) glycerol, 1 mM dithioerythritol, 0.04% (w/v) Triton X-100 and concentrated with detergent exchange as in [8] using hydroxyapatite (HTP, Bio-Rad). The enzyme was obtained in the same buffer solution as the hormone-sensitive lipase.

In the phosphorylation and activation experiments both enzymes were transferred to 5 mM imidazole HCl, pH 7.0, containing 1 mM dithioerythritol, 0.1 M NaCl, 50% (w/v) glycerol, 2 mM  $\text{C}_{12}\text{E}_8$  and 0.05% (w/v) bovine serum albumin, using a micro centrifuge desalting technique [14].

The catalytic subunit of cAMP-dependent protein kinase was purified to homogeneity from rat adipose tissue [15].

### Enzyme Assay

Unless otherwise stated, hormone-sensitive lipase and cholesterol ester hydrolase were assayed against the various substrates as in [8], except that 60 mM potassium phosphate pH 7.0 was used as buffer.

### Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulphate (SDS-PAGE)

This was performed as described by Neville [16]. The following proteins were used as molecular weight markers: glycogen phosphorylase (94 000), transferrin (76 700), bovine serum albumin (67 000), catalase (60 000), ovalbumin (43 000), soy bean trypsin inhibitor (21 000), pancreatic colipase (11 000) and insulin,  $\beta$ -chain (3500). Autoradiography, fluorography and densitometric scanning was carried out as described in [8].

### Peptide Mapping after Limited Proteolysis

Gel slices containing [ $^3\text{H}$ ]DFP-labelled hormone-sensitive lipase and cholesterol ester hydrolase, respectively, were obtained after SDS-PAGE and subjected to peptide mapping by a second SDS-PAGE after limited proteolysis according to Cleveland et al. [17] using *Staphylococcus aureus* V8 protease (0.01  $\mu\text{g}$  per sample).

## RESULTS

### Substrate Specificity

Hormone-sensitive lipase hydrolyses triacyl-, diacyl- and monoacylglycerols and also cholesterol esters [8]. The adrenal cortex cholesterol ester hydrolase is also active against these substrates. However, the presence of a distinct monoacylglycerol lipase (Fredrikson, G., unpublished observation) in the cholesterol ester hydrolase preparation interferes with accurate activity determinations against the acylglycerol substrates (cf [8]). Therefore monoacyl- and diacylalkylglycerol, ether analogues of diacyl- and triacylglycerol respectively were used to compare the relative activities of the two enzymes as, upon hydrolysis, these analogues do not provide substrate for the monoacylglycerol lipase [8,11]. The relative rate of hydrolysis of the different substrates was very similar for the two enzymes (Table 1), when assayed under identical conditions.

### Inhibition Characteristics

The activity of hormone-sensitive lipase is inhibited by micromolar concentrations of DFP and mercuric ions and by millimolar levels of NaF [8]. Cholesterol ester hydrolase is also inhibited by these reagents (Table 2); moreover, at a fixed concentration of inhibitor, the activity of both enzymes is reduced to the same extent. Inhibition of hormone-sensitive lipase by NaF has been shown previously to depend on the specific substrate used [8].

Inhibition of the activity of enzymes by DFP results from covalent modification of the active site [18]; thus labelling

Table 1. Activities of cholesterol ester hydrolase and hormone-sensitive lipase towards different substrates

Enzyme diluted to a detergent concentration of 5  $\mu\text{M}$  was assayed against the substrates for 30 min at 37 °C. Results are means  $\pm$  SEM,  $n = 4-5$ , values expressed relative to activity toward monooleoylalkylglycerol

| Substrate               | Relative activity           |                          |
|-------------------------|-----------------------------|--------------------------|
|                         | cholesterol ester hydrolase | hormone-sensitive lipase |
| Monooleoylalkylglycerol | 100 $\pm$ 1.2               | 100 $\pm$ 3.4            |
| Cholesterol oleate      | 10 $\pm$ 0.1                | 14 $\pm$ 0.3             |
| Dioleoylalkylglycerol   | 5 $\pm$ 0.2                 | 5 $\pm$ 0.1              |

Table 2. Effect of different inhibitors on enzyme activity of cholesterol ester hydrolase (CEH) and hormone-sensitive lipase (HSL)

Enzymes were preincubated at the indicated concentration of inhibitor for 30 min at 37 °C. An equal volume of substrate was then added and the incubation continued for another 30 min. Results are mean  $\pm$  SEM,  $n = 3-5$ , expressed as a percentage inhibition of control

| Inhibitor                           | Monooleoylalkylglycerol |              | Cholesterol oleate |              |
|-------------------------------------|-------------------------|--------------|--------------------|--------------|
|                                     | CEH                     | HSL          | CEH                | HSL          |
| "                                   |                         |              |                    |              |
| DFP, 10 $\mu\text{M}$               | 21 $\pm$ 3.1            | 34 $\pm$ 2.7 | 48 $\pm$ 2.8       | 47 $\pm$ 7.2 |
| NaF, 3 mM                           | 46 $\pm$ 4.4            | 56 $\pm$ 0.8 |                    |              |
| NaF, 30 mM                          |                         |              | 34 $\pm$ 0.9       | 35 $\pm$ 2.6 |
| HgCl <sub>2</sub> 7.5 $\mu\text{M}$ | 46 $\pm$ 1.4            | 49 $\pm$ 1.6 | 39 $\pm$ 1.5       | 46 $\pm$ 0.6 |

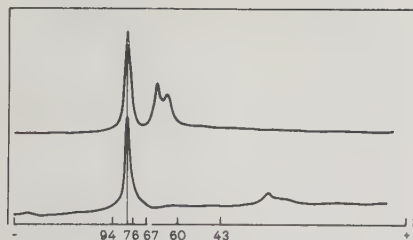


Fig. 1. [ $^3\text{H}$ ]DFP-labelling of hormone-sensitive lipase and cholesterol ester hydrolase. Enzymes were incubated in  $70\ \mu\text{M}$  [ $^3\text{H}$ ]DFP ( $14 \times 10^3\ \text{dis.} \times \text{min}^{-1} \times \text{pmol}^{-1}$ ) for 30 min at  $37^\circ\text{C}$ . Incubations were terminated by protein precipitation in 30% (v/v) acetone followed by 9% (w/v) trichloroacetic acid and treated as in Materials and Methods for SDS-PAGE. Densitometric tracings of fluorographs from SDS-PAGE slab gels. Upper, hormone-sensitive lipase; lower, cholesterol ester hydrolase. The molecular weights ( $\times 10^{-3}$ ) of reference proteins are indicated

with [ $^3\text{H}$ ]DFP allows identification of the enzyme polypeptides. The preparations of both cholesterol ester hydrolase and hormone-sensitive lipase show the presence of one major radioactive polypeptide of  $M_r = 84000$  following treatment with [ $^3\text{H}$ ]DFP (Fig. 1). In each case the polypeptide has been identified previously as the relevant enzyme protein [8,10]. The two enzyme polypeptides have identical mobilities during SDS-PAGE (Fig. 1), indicating that they have identical subunit molecular weights.

#### Phosphorylation and Activation

An important characteristic of hormone-sensitive lipase is that it can be phosphorylated by cyclic-AMP-dependent protein kinase and that phosphorylation leads to an increase in the activity of the enzyme. This is illustrated in Fig. 2 and in Table 3. Previously it has not been possible to investigate directly the ability of cyclic-AMP-dependent protein kinase to phosphorylate cholesterol ester hydrolase due to the lack of homogeneous preparations of the enzyme and to the inability to identify the enzyme polypeptide in an impure preparation.

The identification of cholesterol ester hydrolase as a polypeptide of  $M_r = 84000$  [10] now allows investigation of its possible phosphorylation. The  $84000\text{-}M_r$  polypeptide is indeed phosphorylated by the catalytic subunit of cyclic-AMP-dependent protein kinase (Fig. 2), resulting in activation of the cholesterol ester hydrolase (Table 3). Under the assay conditions employed, the hormone-sensitive lipase and cholesterol ester hydrolase are activated to a similar extent by phosphorylation by cyclic-AMP-dependent protein kinase. The preparation of cholesterol ester hydrolase used in the present study contains several other polypeptides which also become phosphorylated and we are not yet able to exclude completely the possibility that phosphorylation of one of these components is also associated with activation of the enzyme. However, such a possibility seems unlikely.

#### Peptide Mapping

To demonstrate further that the two enzyme polypeptides are structurally similar, we have carried out studies using limited proteolysis of the [ $^3\text{H}$ ]DFP-labelled enzymes. The

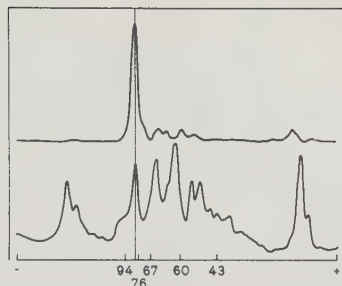


Fig. 2. Phosphorylation of hormone-sensitive lipase and cholesterol ester hydrolase. Enzymes were transferred (Materials and Methods) to a solution containing 5 mM imidazole/HCl, pH 7.0, containing 1 mM dithioerythritol, 0.1 M NaCl, 50% (w/v) glycerol, 2 mM  $\text{C}_{12}\text{E}_8$ , 0.05% (w/v) bovine serum albumin, immediately prior to phosphorylation with the purified catalytic subunit of cAMP-dependent protein kinase from rat adipose tissue in the presence of 5 mM  $\text{MgCl}_2$ , 0.1 mM [ $\gamma\text{-}^{32}\text{P}$ ]ATP ( $10^5\ \text{counts} \times \text{min}^{-1} \times \text{pmol}^{-1}$ ), for 30 min at  $37^\circ\text{C}$ . Phosphorylation was terminated by protein precipitation in 30% (v/v) acetone followed by 9% (w/v) trichloroacetic acid and treated as in Materials and Methods for SDS-PAGE. Densitometric tracings of autoradiography from SDS-PAGE slab gels. Upper, hormone-sensitive lipase; lower, cholesterol ester hydrolase. The molecular weights ( $\times 10^{-3}$ ) of reference proteins are indicated

Table 3. Activation of hormone-sensitive lipase and cholesterol ester hydrolase

Activation of enzymes was performed as for phosphorylation (Fig. 2), except that unlabelled ATP was used and activations were terminated by addition of three volumes of ice-cold 5 mM EDTA, 1 mM dithioerythritol, pH 7.0. Enzyme activity was then assayed against 1.2 mM 1,3-di[ $^3\text{H}$ ]oleoyl-2-O-oleylglycerol in 20 mM Tris HCl, pH 8.0, 5 mM NaCl for 30 min at  $37^\circ\text{C}$ . In controls, ATP was omitted. Probability was determined by Student's *t*-test for unpaired samples. Results are mean  $\pm$  SEM,  $n = 5$

| Enzyme                      | Treatment | Enzyme activity<br>$\text{nmol} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$ | Activation<br>% | <i>P</i> |
|-----------------------------|-----------|-----------------------------------------------------------------------------|-----------------|----------|
| Hormone-sensitive lipase    | control   | $11.0 \pm 0.4$                                                              | 0               | <0.001   |
|                             | activated | $16.3 \pm 0.2$                                                              | 48              |          |
| Cholesterol ester hydrolase | control   | $8.1 \pm 0.3$                                                               | 0               | <0.001   |
|                             | activated | $11.6 \pm 0.2$                                                              | 43              |          |

results of a preliminary study (Fig. 3) show that the patterns of radioactive proteolytic fragments obtained from the two enzymes indeed exhibit similarities. A radioactive polypeptide of  $M_r = 44000$  and those of  $M_r$  below approximately 30000 appear to be common to the two enzymes. This suggests that the two enzymes are structurally related, or indeed identical, but further experiments are necessary to establish this point.

#### DISCUSSION

The data presented here provide strong evidence that the cytosolic cholesterol ester hydrolase activity of bovine adrenal cortex is catalysed by a protein identical to, or very similar to,

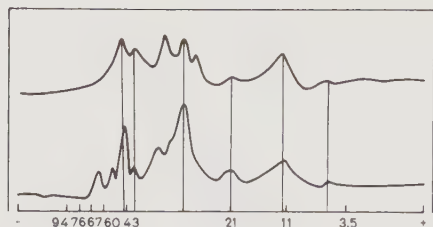


Fig. 3. Peptide mapping after limited proteolysis of [ $^3\text{H}$ ]DFP-labelled hormone-sensitive lipase and cholesterol ester hydrolase. Enzymes were incubated with [ $^3\text{H}$ ]DFP as described in Fig. 1 and subjected to SDS-PAGE. Gel slices containing the enzymes were cut out and the enzyme proteins were subjected to peptide mapping after limited proteolysis (Materials and Methods) by SDS-PAGE (10–22  $\times$  0.9% linear polyacrylamide gradient). Densitometric scans of fluorographs. Upper, hormone-sensitive lipase; lower, cholesterol ester hydrolase. The molecular weights ( $\times 10^{-3}$ ) of reference proteins are indicated

the hormone-sensitive lipase previously identified and characterised in adipose tissue. The lipase which has been purified to near homogeneity from rat adipose tissue is known to hydrolyse cholesterol esters in addition to triacylglycerol [8].

The possible identity of the adrenal cortex cholesterol ester hydrolase and the adipose tissue hormone-sensitive lipase is based on several lines of evidence, resulting from direct comparison of the properties of the two enzymes.

(a) The two enzymes have almost identical substrate specificities, as determined *in vitro* against a range of substrates (Table 1). The actual substrate *in vivo* will, of course, be determined at least partly by the levels of the natural substrate in the particular cell type. In the adipocyte the stored lipids are almost completely made up of triacylglycerol, with relatively little cholesterol esters present, whereas adrenal cortex contains approximately equal amounts of cholesterol esters and triacylglycerol [19]. The functional role of the triacylglycerol in adrenal cortex is not yet clear.

In common with other well-characterised lipases such as lipoprotein lipase, hepatic lipase and monoacylglycerol lipase [20–22], the specific activity of both enzymes is high, being in the range of  $\mu\text{mol}$  fatty acids released  $\times \text{min}^{-1} \times \text{mg}$  protein $^{-1}$ , using triacylglycerol as substrate. The specific activity of the adipose tissue enzyme has been measured directly, using a highly purified preparation [8], whereas the specific activity of the adrenal cortex enzyme has been estimated indirectly from the specific activity of a partially purified fraction [10].

(b) Both enzymes are inhibited by certain selective inhibitors. These include NaF,  $\text{HgCl}_2$  and DFP. At an inhibitor concentration causing approximately half-maximal inhibition, both enzymes are inhibited to a similar degree.

(c) In each case the native enzyme is found in the cytosol in a large protein-lipid complex which can be dissociated by treatment with non-ionic detergents [6,8,23]. Both enzymes have a subunit  $M_r$  of 84 000, as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate [8,10]. Previously, the adrenal enzyme had been reported as having a subunit  $M_r$  of 41 000 [24] but the specific activity of that preparation was very low. In that study the enzyme was purified only 57-fold, whereas it has been estimated that 15 000-fold purification will be necessary to achieve homo-

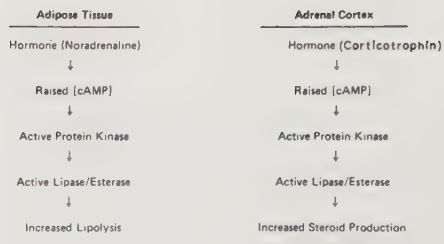


Fig. 4. Outline scheme for hormonal stimulation of lipolysis and of steroidogenesis

geneity [10]. Furthermore, when a 60-fold purified preparation of the enzyme, in which a polypeptide of  $M_r = 41\,000$  was the major component, was incubated with [ $^3\text{H}$ ]DFP to inactivate the enzyme, no radioactivity was incorporated into the 41 000- $M_r$  component. Under these conditions only one polypeptide, of  $M_r = 84\,000$ , became radiolabelled [10]. Interestingly, it has also been reported that hormone-sensitive lipase from chicken adipose tissue has a subunit  $M_r$  of 42 000–45 000, but again the specific activity of that preparation was extremely low [25] and its relation to hormone-sensitive lipase has been questioned [8].

(d) Both enzymes are substrates *in vitro* for the cyclic-AMP-dependent protein kinase (Fig. 2), phosphorylation of the enzyme causing activation. The extent of activation is similar for both enzymes when assayed against the triacylglycerol ether analogue (Table 3). The demonstration that the catalytic subunit of cyclic-AMP-dependent protein kinase phosphorylates and activates the adrenal cortex enzyme is the first direct evidence that the cholesterol ester hydrolase is indeed regulated by covalent phosphorylation. It has been shown previously by several workers that the enzyme was activated under conditions favouring phosphorylation [3,24,26]. This was accompanied by incorporation of phosphate into trichloroacetic-acid-precipitable material but as only partially purified preparations of the enzyme were used, it was not possible to show that activation resulted from phosphorylation of the actual enzyme protein. The identification of the enzyme protein [10] allows the demonstration that the cholesterol ester hydrolase is indeed phosphorylated by the catalytic subunit of cyclic-AMP-dependent protein kinase. Phosphorylation of the adrenal cortex cholesterol ester hydrolase by cyclic-AMP-dependent protein kinase does not, of course, preclude the possibility that the enzyme is also regulated by other cyclic-AMP-independent protein kinases. This requires investigation.

(e) The two enzymes exhibit a similar pattern of radioactive fragments after limited proteolysis of the [ $^3\text{H}$ ]DFP-labelled enzymes (Fig. 3). Although further experimentation is necessary, the similarities between the two degradation patterns provide additional support for the conclusion that the enzymological similarities of the two enzymes have a common structural basis.

The results presented here provide the first demonstration of the existence of hormone-sensitive lipase in a tissue other than adipose tissue. In adrenal cortex the main function of the enzyme appears to be to serve as a cholesterol ester hydrolase, releasing cholesterol to be used for the synthesis of steroids. Corpus luteum, another major steroid-producing organ, also contains an activatable cholesterol ester hydrolase [27] and

it will be of interest to investigate whether that activity is also catalysed by hormone-sensitive lipase.

Our findings suggest an interesting extension to the scheme proposed by Edwin Krebs to explain the actions of hormones which act through raising the intracellular levels of cyclic AMP [28]. He proposed that several hormones act on metabolic systems in different tissues by causing different proteins to become phosphorylated, altering their functional properties and resulting in the observed physiological effect. In the cases considered here, two apparently unrelated metabolic systems in two different organs, namely adipose tissue lipolysis and steroid production in adrenal cortex, are shown to be regulated hormonally by phosphorylation of the same enzyme protein, resulting in different physiological effects (Fig. 4).

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## REFERENCES

- Boyd, G. S. & Gorman, A. M. S. (1980) *Mol Aspects Cell. Regul.* **1**, 95–134.
- Trzeciak, W. H. & Boyd, G. S. (1973) *Eur. J. Biochem.* **37**, 327–333.
- Trzeciak, W. H. & Boyd, G. S. (1974) *Eur. J. Biochem.* **46**, 201–207.
- Gorman, A. M. S. & Boyd, G. S. (1977) *FEBS Lett.* **79**, 54–58.
- Pittman, R. C. & Steinberg, D. (1977) *Biochim. Biophys. Acta*, **487**, 431–444.
- Yeaman, S. J., Cook, K. G. & Lee, F.-T. (1980) *FEBS Lett.* **120**, 212–216.
- Belfrage, P., Jergil, B., Strålfors, P. & Tornqvist, H. (1977) *FEBS Lett.* **75**, 259–264.
- Fredrikson, G., Strålfors, P., Nilsson, N. Ö. & Belfrage, P. (1981) *J. Biol. Chem.* **256**, 6311–6320.
- Fredrikson, G., Strålfors, P., Nilsson, N. Ö. & Belfrage, P. (1981) *Methods Enzymol.* **71**, 636–646.
- Cook, K. G., Lee, F.-T. & Yeaman, S. J. (1981) *FEBS Lett.* **132**, 10–14.
- Tornqvist, H., Björgell, P., Krabisch, L. & Belfrage, P. (1978) *J. Lipid Res.* **19**, 654–656.
- Nilsson, Å., Nordin, H. & Wilhelmsson, L. (1973) *Biochim. Biophys. Acta*, **296**, 593–603.
- Chang, K.-J., Marcus, N. A. & Cuatrecasas, P. (1974) *J. Biol. Chem.* **249**, 6854–6865.
- Helmerhorst, E. & Stokes, G. B. (1980) *Anal. Biochem.* **104**, 130–135.
- Strålfors, P. & Belfrage, P. (1982) *Eur. J. Biochem.* in the press.
- Neville, D. M., Jr (1971) *J. Biol. Chem.* **246**, 6328–6334.
- Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) *J. Biol. Chem.* **252**, 1102–1106.
- Cohen, J. A., Oosterbaan, R. A. & Berends, F. (1967) *Methods Enzymol.* **11**, 686–702.
- Christie, W. W. (1981) in *Lipid Metabolism in Ruminant Animals* (Christie, W. W., ed.) pp. 95–191, Pergamon Press, Oxford.
- Bengtsson, G. (1979) Thesis, Umea University Medical Dissertation 48, Umea University.
- Kuusi, T. (1979) Thesis, Helsinki University.
- Tornqvist, H. & Belfrage, P. (1976) *J. Biol. Chem.* **251**, 813–819.
- Lee, F.-T. & Yeaman, S. J. (1980) *Biochem. Soc. Trans.* **8**, 728–729.
- Beckett, G. J. & Boyd, G. S. (1977) *Eur. J. Biochem.* **72**, 223–233.
- Berglund, L., Khoo, J. C., Jensen, D. & Steinberg, D. (1980) *J. Biol. Chem.* **255**, 5420–5428.
- Naghsheini, S., Treadwell, C. R., Gallo, L. L. & Vahouny, G. V. (1978) *J. Lipid Res.* **19**, 561–569.
- Bisgaier, C. L., Treadwell, C. R. & Vahouny, G. V. (1979) *Lipids*, **14**, 1–4.
- Krebs, E. G. (1972) *Curr. Top. Cell. Regul.* **5**, 99–133.

K. G. Cook and S. J. Yeaman, Department of Biochemistry, University of Newcastle-upon-Tyne, Ridley Building, Newcastle, Great Britain, NE1 7RU

P. Strålfors, G. Fredrikson, and P. Belfrage, Medicinska-Kemiska Institutionen 4, Lunds Universitet, Box 750, S-220 07 Lund, Sweden











